PATHOGEN-INDUCED PLANT CHEMICAL DEFENSE: EFFECT ON INSECT HERBIVORES AND PARASITOIDS

By

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I dedicate this work to Alonso, my husband and best friend. I thank him for being my pillar of strength and my endless source of inspiration. I would not have made it this far without all his love and support.

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Abstract of Thesis Presented to the Graduate School
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PATHOGEN-INDUCED PLANT CHEMICAL DEFENSE: EFFECT ON INSECT HERBIVORES AND PARASITOIDS

By

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The study presented here is the first in which the production of volatile compounds by a single host-plant system in response to both insect herbivores and pathogens has been evaluated simultaneously. Additionally, it is the first study on the effect of previous pathogen infection on the production of plant volatiles in response to insect damage. The study of plant volatile defenses may improve our understanding of plant resistance mechanisms to disease and insect herbivores. The identification of specific pathogen- and herbivore-induced plant volatiles will greatly contribute to the development, improvement and implementation of host-plant resistance and other control methods for insect and pathogen pests.

In my peanut-white mold-beet armyworm system, beet armyworm (BAW) feeding and oviposition preference and larval performance were all enhanced by the fungal- infection on peanut plants. This may indicate an interference of the fungal

infection with the plant's direct chemical defenses against the herbivores. Peanut plants release a specific set of volatile chemical in response to white mold infection. This volatile profile differs qualitatively and quantitatively from signals emitted in response to BAW damage. Previous infection on the plant by the white mold fungus does not interfere with the emission of volatiles by the infected plant in response to BAW attack. The white mold-derived compound 3-octanone and the plant-produced volatile methyl salicylate were only recovered from plants infected with *S. rolfsii*. Thus, the presence of these compounds could potentially be used in the future for the detection of white mold-infected plants in the field. Although BAW preference and performance were enhanced by white mold infection on peanut plants, it does not appear that previous infection of the plant has any negative effect on the host searching ability of its parasitoid, *Cotesia marginiventris*. Parasitoids were actually observed landing more frequently on, and were significantly more responsive to volatiles from infected, rather than healthy peanut plants. This may be a response to higher amounts of volatiles being released by infected plants either caused by the plant's increased sensitivity to BAW feeding due to the fungal infection or to increased feeding by the BAW on leaves from infected plants.

Finally, I found that pepper plants under simultaneous attack by leaf spot bacteria, *Xanthomonas campestris* pv. *vesicatoria* (pepper race 3) (XCVP3) and BAW were able to produce a volatile profile qualitatively similar to, but quantitatively greater than, that produced by plants under insect damage alone during the 4 days after bacterial inoculation. However, after 4 days from bacterial inoculation, the amount of volatiles emitted by XCVP3-infected plants in response to BAW infestation was significantly lower than that produced by healthy plants in response to BAW damage. Analyses of the

levels of signaling hormones jasmonic acid (JA) and salicylic acid (SA) in these plants revealed that the reduction in volatile production in response to insect damage was preceded by a significant increase in conjugated SA, and consequent release of methyl salicylate by plants under the combined bacterial/insect attack. Also, the cumulative JA increase in XCVP3/BAW plants was significantly higher than that of control plants, but did not differ significantly from those of plants damaged by either organism alone. Overall, the data presented herein support the idea of an orchestrated interaction between the levels of JA and SA to regulate the induction and emission of volatile organic compounds by pepper plants under bacterial and insect herbivore attack.

CHAPTER 1
LITERATURE REVIEW

Introduction

Herbivorous insects and plant diseases are serious threats for the agricultural environment because they reduce yield and quality of crops. In particular, plant diseases (caused by infectious viruses, bacteria, phytoplasmas, fungi, and nematodes) cause serious losses in agricultural commodities. These problems include reduced yields, lower product shelf-life, and decreased aesthetic and nutritional value. In addition to the direct damage to the plant, some pathogen strains and insect species also result in the production and accumulation of secondary metabolites and toxins that can cause health problems in humans and animals. Control of plant diseases and pests is vital for providing an adequate supply of food, feed, and fiber to cope with the increasing human population and its demands. In Florida alone, more than a dozen plant disease epidemics occurred from 1970-1990 (Kucharek 1990). Growers currently spend large sums of money to control pathogens and insects that attack their crops. Nevertheless, crop and commodity losses because of diseases and herbivore damage cost billions of dollars each year. In the United States, it is estimated that the yearly economic losses are approximately 9.1 billion dollars for plant diseases, and approximately 7.7 billion dollars for insect damage, all this after the application of control measures practiced under modern agriculture (Agrios 1997). Thus, information about how insect and pathogen

pests interact with crops, and how this interaction affects the economic value and quality of agricultural products is important for establishing the economic thresholds for managing pest populations, minimizing pest damage, developing new methods of insect and pathogen prevention and control, and improving host-plant resistance and other mechanisms for tolerance to insect and pathogen pests.

Plant Chemical Defenses Against Insect and Pathogens

Plants play an active role in the interactions taking place in their ecosystem. Plants possess a number of chemical defense mechanisms that are often triggered by herbivore and/or pathogen attack. The defense mechanisms of plants can be structural, morphological, or chemical (Kessman et al. 1994, Sticher et al.1997). Morphological and structural features such as leaf and flower size, shape and color, presence of trichomes, cell wall thickness, thickness of the wax layer, and even the texture of cuticle may cause certain insects to avoid a plant (Painter 1958). These structural and morphological barriers provide the first line of resistance in the plant's defense against many potential invaders.

In addition to structural and morphological defenses, plants also possess chemical defenses that are effective once the attacking organisms have made it past the first line of defense. For example plants may contain significant amounts of constitutive secondary metabolites including phenolics, terpenoids, and steroids, which are toxic to invading organisms (Levin 1976, Mauch-Mani & Métraux 1998). Some plant compounds which are also actively produced or induced after penetration of the tissues has occurred. These inducible defenses are energy-costly and are only produced after specific recognition of the invading organism. Plants that are able to recognize an invading organism activate

responses including a rapid localized cell death, also known as hypersensitive response, at the site of penetration and activation of biochemical defense responses. Biochemical responses include the production of reactive oxygen species, structural changes in the cell wall, accumulation of defense-related proteins and phytoalexin biosynthesis (Benhamou 1996, Dixon 1986, Hammerschmidt 1999). These chemical defenses can directly modify the development and survival of the attacking organism (i.e., phytoalexins, proteinase inhibitors) (Dixon 1986, Mür et al. 1997).

Plant Volatile Production in Response to Insect and Pathogen Attack

In addition to the production of internal defense compounds in response to pest attack, plants may also produce volatile substances that are released externally. Plants release a mixture of such compounds in response to attack by herbivores (McCall et al. 1994, Loughrin et al. 1995, Röse et al. 1996, Paré and Tumlinson 1997). These chemical signals are attractive to parasitoids of the pests (Röse et al. 1998; Turlings et al. 1991, 1993), and since both the emitter (plant) and the recipient (parasitoid) benefit from these infochemicals, they are categorized as synomones (Nordlund 1981). In addition to direct herbivore feeding, some plants also release synomones in response to herbivore oral secretions or regurgitate when this is either applied topically on a mechanically-damaged leaf or fed through the stem of excised plants (Boland et al. 1995, Alborn et al. 1997).

Pathogens can also elicit the production and release of volatiles from the affected plant hosts. Case in point, *Brassica rapa* seedlings were found to release volatile products of glucosinolate degradation when infected by the fungus *Alternaria brassicae* (Doughty et al. 1996). In this study, compounds such as dimethyl disulphide, dimethyl trisulphide, 3-butenyl and 4-pentenyl isothiocyanates, and 4-oxoisophorone were emitted

in larger amounts or exclusively from plants infected with the fungus, 9-d after inoculation, than from healthy uninoculated control plants. In another study with beans (*Phaseolus vulgaris* L.) release of volatile linolenic acid derivatives, such as cis-3-hexenol and trans-2-hexenal, ensued 15-24 h post-inoculation with *Pseudomonas syringae* pv. *phaseolicola* (Croft et al. 1993). Both of the compounds released by infected bean plants in this study are C_6 aldehydes which are believed to serve as wound-induced volatile signals, turning on the plant's defense system (Bate & Rothstein, 1998). Lipoxygenase-derived volatiles are also emitted from discs of pepper, *Capsicum annuum* L., leaves 2-12 h after infiltration with a virulent strain of the bacterial pathogen *Xanthomonas campestris pv. vesicatoria* (Buonaurio and Servili, 1999). Compounds evolved from this system included (E, E)-2, 4-hexadienal, 1-hexanol, 3-hexen-1-ol, 2, 4 hexadienal, and 2, 4-heptadienal.

Biochemical Pathways in Plant Defense Against Insect and Pathogen Pests

Different plant defense pathways are generally induced in response to insect herbivores and pathogen attack. Jasmonic acid causes expression of defense compounds such as proteinase inhibitors and polyphenol oxidases, which provide plant resistance against insects (Ryan 1990, Farmer & Ryan 1990, Farmer et al. 1992, Thaler et al. 1999). Salicylic acid (SA), on the other hand, is involved in the production of pathogenesis-related proteins and phytoalexins in response to pathogen infection.

Lipid-mediated signaling in plants is an area of research that has grown rapidly in recent years. Compounds derived from linoleic and linolenic acids are of particular interest because of their role as regulators of plant development and defense processes (Howe 2001). Oxylipins are oxygenated fatty acids, products of the lipoxygenase (LOX)

pathway, and have been directly linked to direct plant defense responses to wounding and damage by herbivorous insects (Ryan 1990, Farmer & Ryan 1990, Farmer et al. 1992 Doares et al. 1995). Jasmonic acid is an oxylipin, derived from oxidation of linolenic acid, which activates genes that induce production of proteinase inhibitors and phytoalexins in response to wounding (reviewed in Choi et al. 1994, Staswick et al. 1993). Increase in JA levels has been reported in cell cultures exposed to fungal elicitors (Gundlach et al. 1992). Jasmonic acid and its methyl ester also directly induce proteinase inhibitors production when applied to plants exogenously (Farmer & Ryan 1990, Ryan 1992).

Generally, plant defense responses mediated by products of the LOX pathway are specific and affect the invading organism in particular. There is another type of induced resistance referred to as systemic acquired resistance (SAR), which is temporally and spatially separated from the site of the initial pest attack on the plant. Systemic acquired resistance is the activation of a broad spectrum of host defense mechanisms. Systemic acquired resistance is generally effective against a wide range of pathogens, including viruses, bacteria and fungi, and lasts for several weeks or even months. Systemic acquired resistance can be activated locally, at the site of the initial pathogen attack, and/or systemically, in tissues away from the site of infection (Delaney et al. 1994). Studies conducted with transgenic tobaccok, *Nicotiana tabacum*, and *Arabidopsis* plants expressing the bacterial NahG gene, which causes the plant to produce salicylate hydroxylase that converts SA to catechol, have provided evidence for the key role of SA in inducing SAR (Delaney et al. 1994). This conversion prevents these plants from inducing SAR when infected with a pathogen (Gaffney et al. 1993). The methyl ester of

SA is also believed to play an important role in the plant defense signaling cascade. For example, Shualev et al. (1997) found that methyl salicylate was released by tobacco plants infected with a virus and that this compound was capable of inducing expression of PR-proteins in plants.

Another mediator of plant defense responses is the highly volatile plant hormone, ethylene. The production of ethylene in plants is induced by various factors such as, mechanical wounding, exogenous auxin applications, and herbivore and pathogen attack. Chaudhry et al. (1998) found that the production and release of ethylene increased in tobacco plants 48 h post-inoculation with cucumber mosaic virus (yellow strain). Additionally, it was observed that the increase in ethylene was positively correlated with an increase in the concentration of the enzymes required for its synthesis (Chaudhry et al. 1998). Ethylene interacts with SA in inducing the expression of pathogenesis-related proteins involved in the induction of SAR. Ethylene, in conjunction with SA, is also believed to be responsible for the localized hypersensitive response of plants to pathogen attack by contributing to the development of necrotic local lesions that prevent the pathogen from spreading on to adjacent healthy tissues (reviewed in Chaudhry et al. 1998, O'Donnell et al. 2001). O'Donnell et al. (2001) found that ethylene production and perception was in fact necessary for SA accumulation in tomato, *Lycopersicon esculentum*, plants infected with *Xanthomonas campestris* pv. *vesicatoria*. Jasmonic acid and ethylene also interact in the induction of a special kind of resistance, termed induced systemic resistance (ISR), induced in plants by non pathogenic rhizobacteria (Chang and Shockey 1999, Ton et al. 2002). Induced systemic resistance confers resistance to the rhizobacteria-colonized plant against pathogens and insects. In response to insect damage and pathogen infection, ethylene also acts synergistically with JA to induce wound response genes leading to production of proteinase inhibitors and PR-proteins (Maleck & Dietrich 1999, O'Donnell et al. 1996, Xu et al. 1994).

Expression of wound- and JA- inducible genes can be regulated by ethylene or SA. For example, JA induction of proteinase inhibitor gene expression in tomato and soybean requires ethylene (O'Donnell 1996). On the other hand, the expression of thionins and lectin II in certain plants can be induced by JA and suppressed by ethylene (O'Donnell 1996, Van Loon 1997). Recent studies have also suggested conflicting interactions between the JA and SA pathways. For example, beet armyworm (BAW), *Spodoptera exigua*, and corn earworm, *Helicoverpa zea*, have been reported to feed more upon tomato plants that had been previously treated with an elicitor of systemic acquired resistance, which is regulated by SA (Stout et al. 1999). Tomato plants treated with this SAR-elicitor were found to have reduced expression of genes encoding proteinase inhibitors (Fidantsef et al. 1999). On the other hand, use of the same SAR-elicitor on cotton had no impact whatsoever on the feeding preference and performance of whiteflies (*Bemisia tabaci*)and cotton bollworms, *Spodoptera armigera* (Inbar et al. 2001). It would therefore appear that JA, SA and ethylene are all key players in inducing and regulating plant defenses against plant pathogens and insect herbivores. However, the interactions involved among these signaling compounds need to be further investigated to determine the extent of their positive, negative, and/or synergistic interaction in the plant's defense against different pest organisms. The induction and release of volatiles by plants raise questions about the processes involved in the induction and production of such chemicals. It is not yet clear what defense pathways are involved in the production of volatile chemicals by plants in response to insect and pathogen attack. However, four major biosynthetic pathways are believed to be involved in the production of plant volatiles in response to damage by lepidopterous larvae (Paré & Tumlinson 1997). The shikimic/ tryptophan, mevalonate, Rohmer, and LOX pathways are all believed to be involved in the production of the different classes of volatile compounds released by plants under attack by insect herbivores (Paré & Tumlinson 1997). In fact, the LOX pathway product JA and its methyl ester have been found to induce volatile emissions similar to those

resulting from herbivory in many plants (Boland et al. 1995, Dicke et al. 1999). It remains to be elucidated how plant indirect defenses against herbivores, consisting of volatile compounds, are influenced by the presence of phytopathogens, and how simultaneous attack of insects and phytopathogens on the same plant may affect the plant's production of volatile compounds. Recent studies suggest that activation of plant internal defenses by pathogens interferes with plant defenses against herbivorous arthropods and vice-versa (Karban et al. 1987, Fidantsef et al. 1999, Stout et al. 1999, Bostock 1999, Felton et al. 1999). However, whether the same negative interactions exist in the pathways involved in plant volatile responses to pathogens and herbivorous insects remains to be determined.

Insect- and Pathogen-Derived Elicitors of Plant Defense Responses

The substances derived from the attacking organisms that can induce defense responses when applied to plants, plant tissues, or plant cell cultures are termed elicitors (Alborn et al. 1997, Hammerschmidt 1999, Mattiacci et al. 1995, Dixon 1986, Weiler 1997). These elicitor compounds are capable of inducing activation of plant defense responses even in the absence of the living pest (Alborn et al. 1997, Dixon 1986, Gundlach et al. 1992, Mattiacci et al. 1995).

Unlike pathogen-produced elicitors, insect-produced elicitors are relatively rare. However, a couple of compounds responsible for eliciting the emission of plant volatiles have been isolated and identified in recent years. These insect-derived elicitors are volicitin, a component found in *Spodoptera exigua* regurgitant (Alborn et al. 1997) which elicits volatile production in corn, and beta-glucosidase, found in oral secretions of *Pieris bassicae* (Mattiacci et al. 1995) which elicits volatile production in cabbage.

For successful infection of a plant, pathogens depend on a variety of chemicals, such as, cell wall degrading enzymes and toxins. Many of the products secreted by

pathogens may be recognized by plants and thus, act as elicitors of defense responses (Doares et al. 1995, Weiler 1997, Hammerschmidt 1999). Upon recognition of these elicitors, plants may respond by activating the production of phytoalexins, the production of reactive oxygen species, or the accumulation of lignin, callose, and/or proteinase inhibitors (Weiler 1997, Hammerschmidt 1999). Arachidonic acid, a fatty acid found in the fungus *Phytophtora infestans* (potato late blight), has also been found to activate phytoalexin-encoding genes but these are different from those activated by JA in potato discs (Choi et al. 1994). This finding may be an indication that the biochemical pathways involved in plant defense against herbivores are different from those involved in the defense against pathogens. Pathogen derived compounds such as cellulysin, which is a crude cellulose extract from the fungus *Trichoderma viride*, have been found to induce volatile production in tobacco, lima bean, and corn plants (Piël et al. 1997, Koch et al. 1999). Piël et al. (1997) found that a 50 μg/mL concentration of cellulysin elicited the emission of hexenyl acetate, ocimene, linalool, nonatriene, indole, bergamotene, beta-farnescene, nerolidol and tridecatetraene 12-24 h post application to cut petioles. Additionally, they observed that these emissions were similar in nature to those elicited by applications of JA, and that their production could be blocked by inhibitors of the JA pathway. The compounds reported in this experiment have also been reported to be produced by plants in response to insect damage. However, caryophyllene which is a compound induced in corn by insect damage, was not present in the emissions of this plant in response to cellulysin, and bergamottene, another herbivore-induced compound, was only present in relatively small proportions. Similarly, coronatine, a phytotoxin isolated from *Pseudomonas syringae* bacteria, also elicits the release of volatiles in

plants. Coronatine toxicity in plants induces symptoms similar to those observed in plants treated with high doses of JA, such as chlorosis, accelerated senescence, and ethylene release (Weiler et al. 1994, Boland et al. 1995). However, Weiler et al. (1994) argued that coronatine is not a JA analogue because: 1) it actively elicits tendril coiling in *Bryonia* and induces pathogen defense systems in plant cell cultures at lower concentrations than exogenously applied jasmonates, 2) it does not induce accumulation of endogenous JA and, 3) its structure strongly resembles that of 12-oxo-phytodienoic acid (PDA). Because PDA is a known precursor of jasmonates, Weiler et al. (1994) concluded that coronatine elicits plant defense responses because it is a PDA analog.

Interactions between Phytopathogen and Herbivorous Insects

For many years, the study of physiological and chemical changes induced in plants by the attack of insects and pathogens has captured the interest of scientists in the areas of plant pathology and entomology, independently. However, previous insect damage can alter the biochemistry of the plant in such a way that the performance of pathogens is compromised. In the same manner, pathogen infection results in the alteration of the chemical quality of the host plant affecting the feeding preference and performance of phytophagous arthropods.

Host plant selection and feeding preference of insect herbivores is influenced by a number of factors, including feeding stimulants and deterrents within plants. The level of these compounds may vary from plant to plant and can be influenced by stress factors such as pathogen infection (Hatcher 1995). Major nutrients such as carbohydrates and proteins are listed as some of the most important phagostimulants for phytophagous insects (Matsuda 1988) whereas secondary plant defensive compounds such as tannins

(phenolics) and protease inhibitors can have the opposite effect, acting as deterrents (Slansky & Scriber 1985). The effect of fungal infection on insect feeding preference and their performance on plants varies widely. For example, Kluth et al. (2001) found that when given a choice between healthy and rust-infected thistle, chrysomelid beetles preferred to feed on the healthy plants. Also, Hatcher et al. (1995) found that infection of *Rumex* spp. by rust fungi increased feeding preference by the chrysomelid beetle *Gastrophysa viridula*, but larval performance and adult reproductive capacity were negatively affected. In the same study, rust-infected leaves were found to have lower nitrogen and higher oxalate concentration than healthy leaves, which might account for the negative effects observed in the insects. Moran (1998) found that discs from cucumber, *Cucumis sativus*, leaves infected with *Cladosporium* fungi were preferably fed on by cucumber beetles, *Diabrotica undecimpunctata howardii*. Similarly, other studies have found that plant defensive responses against pathogens have a negative effect on the plant's ability to cope with insect herbivory (Bostock 1999, Felton et al. 1999, Fidantsef et al. 1999, Karban et al. 1987, Stout et al. 1999). On the other hand, there have also been reports suggesting enhanced plant defenses against insects in plants after pathogen inoculation. For example, McIntyre et al. (1981) found that infection of tobacco plants by the tobacco mosaic virus increased resistance to green peach aphids, *Myzus persicae*. Direct and indirect interactions between other plant pathogenic fungi and insects species with either negative, positive or neutral outcomes for the insect are reviewed in Hatcher (1995).

Most reports on the effect of insect damage on plants on subsequent pathogen colonization deal with facilitation of pathogen penetration through insect feeding or

oviposition wounds (Herzog et al. 1975, Friedli and Bacher 2001). However, insect damage on the plant can also induce resistance against future pathogen infection (Hatcher 1995). This is the case in *Rumex* spp. where damage by a chrysomelid beetle caused an 80% reduction in pustule density by a rust fungus (Hatcher at al 1994). Similarly, soybean crown rot was negatively affected by plant defoliation by the soybean looper (Padgett et al. 1994). To my knowledge, no studies have been conducted to determine the exact biochemical processes responsible for either the positive or negative effect of insect feeding on pathogen performance on the same host plant. Most of the studies mentioned above focused on inducible direct-defense compounds. Thus, it is still not known how plant indirect defenses against herbivores, consisting of volatile compounds, are influenced by the presence of phytopathogens.

Effect of Induced Plant Volatiles on Pathogen, Insect Herbivores, and Parasitoids

Plant volatiles can affect pathogens in either positive or negative ways. Germination and growth of white mold (*Sclerotium rolfsii*) are stimulated by the release of methanol and other volatile compounds emanating from moist peanut hay (Shokes et al. 1996). On the other hand, volatiles from ground-up healthy corn kernels resistant to *Aspergillus flavus*, have been found to inhibit growth and aflatoxin production in colonies of this pathogen (Zeringue et al. 1996). In cotton, however, the lipoxygenase-derived volatile trans-2-hexenal inhibited, while alpha and beta pinene stimulated the growth of this fungus (Zeringue & McCormick 1989, 1990). Other compounds like 3-methyl-1-butanol and 3-methyl-2-butanol were found to decrease fungal growth but increased aflatoxin production (Zeringue & McCormick 1990). In the case of coronatine-induced volatiles in beans, the compounds emitted were trans-2-hexenal, which had high

bactericidal activity and cis-3-hexenol, which was also bactericidal but only at much higher doses (Croft et al. 1993). Furthermore, these compounds were emitted in larger quantities from resistant varieties compared to susceptible ones (Croft et al. 1993).

Volatile compounds released by insect damaged- or chemically-elicited plants can also serve as cues by which insect herbivores can locate potential host plants. For example, Colorado potato beetle, *Leptinotarsa decemlineata*, adults were attracted to potato plants damaged by conspecific larvae. These insects were equally attracted to potato foliage treated with either regurgitate collected from larvae of Colorado potato beetle or cabbage looper, or with methyl jasmonate (Landolt et al. 1999). On the other hand, volatiles released in response to herbivore attack can also serve as repellents against insects. The odors released by wheat seedlings in response to infestation by a high density of aphids were found to repel conspecific (Quiroz et al. 1998). Also, De Moraes et al. (2001) reported that females of tobacco budworm preferred to oviposit on undamaged tobacco plants instead of those fed on by larvae of their same species. This avoidance was found to be mediated by the odors emitted by the plants in response to larval feeding (De Moraes et al. 2001).

The volatiles produced by plants in response to insect damage act as an indirect defense because they can be used by natural enemies to locate their herbivorous hosts. For example, predatory mites, *Phytoseiulus persimilis,* have been found to be attracted to odor blends emanating from host plants damaged by their prey, *Tetranychus urticae* (Dicke & Sabelis 1988, Dicke et al. 1990). Also, enhanced emission of plant volatile chemicals result from BAW feeding on corn plants (Turlings et al. 1991a). These volatile emissions have been found to attract the parasitoid, *Cotesia marginiventris,* to the micro-

habitat of the herbivores (Turlings et al. 1991a). In fact, herbivore-induced volatile compounds released by plants have been shown to be the most important cues used by parasitoid wasps to locate their host caterpillars (Turlings et al. 1991a, b). Many insect herbivores use the volatile compounds produced by plants to locate their host plants, and natural enemies also use volatile chemicals to locate their herbivorous hosts, therefore the volatiles emitted by plants in response to attack by pathogens may influence oviposition site selection by herbivore females and in the host-searching process by natural enemies (Doughty et al. 1996).

Research Goals

Enhancing resistance to herbivores and disease in plants is an excellent management option and is often very cost-effective and environmentally safe. This approach, however, depends on our ability to identify and characterize the sources of resistance in crop species and in closely related plants. It is now known that plant volatiles play an important role in plant defense against both, herbivorous and pathogenic organisms and thus, may have a significant role in the regulation of the behavior, development, and survival of such organisms. Therefore, the study of the chemically-mediated interactions, and the identification of the chemical compounds involved in this mediation, between pathogen and pests with their host plant species are critical for the development of ecologically sound integrated management programs. Comparison of plant volatile profiles induced by pathogens and insects, and their combination, will give us a better idea, based on the nature of the compounds produced, of whether plant defense against these agents share the same biochemical pathways. Furthermore, the results obtained from such investigation will provide the basis for additional studies on the value

of these plant-derived volatiles as possible antimicrobial agents, and for the identification of plant varieties with an enhanced chemical arsenal against pests.

The overall goal of this project was to investigate the possible production of volatile compounds by plants under pathogen attack, and to evaluate the effect of simultaneous pathogen/herbivore challenge on the volatile emission by host plant. The specific objectives are to: 1) analyze, identify, and compare compounds from head space collections from pathogen-infected and healthy plants, 2) to determine the effect of pathogen defense induction on the production and release of herbivore-induced volatiles by the host plant, 3) to determine the effect of pathogen induced-biochemical changes in plants on herbivorous and parasitoid insects, and 4) to investigate the plant's biochemical defense mechanisms involved in pathogen and herbivore defense.

CHAPTER 2
IN VIVO VOLATILE EMISSIONS FROM PEANUT PLANTS INDUCED BY SIMULTANEOUS FUNGAL INFECTION AND INSECT DAMAGE

Introduction

Plants are attacked by a number of organisms during their life span, including insect herbivores and disease-causing microorganisms. In response, plants have evolved complex defense mechanisms to fend off their parasites. These mechanisms may be triggered by a specific organism, and may be effective against present and future attacks by organisms of the same or different species. This phenomenon is known as cross-resistance and has been the subject of interest for many biologists since the 1970s (Karban & Baldwin 1997). Another field that has received much attention, especially in the last decade, is the induction of what are now called indirect defenses (i.e., volatile emissions) in plants by feeding of herbivorous insects. Induced volatile compounds released by the plant attract natural enemies of the herbivore inflicting the damage (Dicke and Sabelis 1988, Turlings et al. 1991).

It is clear that a thorough understanding of the plant's own defense mechanisms is required for their use against the pests that present a constant threat to our agricultural commodities. Although, in a typical field situation, plants are confronted with a wide array of antagonistic organisms, most studies on induced plant defenses have been conducted using plant-pathogen or plant-herbivore systems independent from one another. However, in recent years, many studies have suggested an interaction (or cross-

talk) between the pathways involved in plant defense to pathogens and herbivorous insects. In many cases it appears that induction of plant defenses by one type of organism interferes with plant defense against the other (Karban et al. 1987, Fidantsef et al. 1999, Stout et al. 1999, Bostock 1999, Felton et al. 1999). Much remains to be learned about how plants defend themselves against multiple stress factors. All studies conducted on this subject have focused on inducible direct defense compounds. It is still not known how plant indirect defenses against herbivores, consisting of volatile synomones, are influenced by the presence of phytopathogens, and how simultaneous attack of insects and phytopathogens on the plant may affect its production of indirect defense substances.

In the present study I tested the effect of peanut, *Arachis hypogaea* L. (Fabaceae), stem infection by the white mold fungus, *Sclerotium rolfsii* Sacc. (mytosporic fungi), on the feeding preference of beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera:Noctuidae). I also evaluated and compared the emission of volatile compounds from peanut plants under attack by the white mold fungus and by beet armyworm. The emission of volatile compounds from *S. rolfsii* fungal cultures was also evaluated. Finally, I tested the effect of commercially available compounds identified from the volatile profile emitted by fungus-infected plants on the radial growth of laboratory cultures of *S. rolfsii*.

Materials and Methods

Plant and Insect Material

'Georgia Green' peanut seeds were provided by Drs. Tim Brenneman and Glen Raines (Coastal Plain Experiment Station, University of Georgia, Tifton, GA). Seeds were sown in pairs in 3.78-L pots (16-cm diam) containing a 1:1 (vol:vol) mixture of

commercially available filter sand and Metromix 300 (Scotts-Sierra Horticultural Company, Marysville, OH). Plants were grown in an insect-free greenhouse with natural light, under Florida summer conditions (14L:10D light cycle). The greenhouse temperature was kept between 25-30°C. After emergence, seedlings were thinned to one individual per pot. Each plant received 100 ml of a 3.38 g/L liquid fertilizer solution (20-20-20 [N-P-K] Peters, W. R. Grace, Fogelsville, PA) every two weeks starting one week after emergence. Five-week old peanut plants with six fully developed leaves on the main stem and three fully developed leaves on each of two secondary branches were used in all experiments.

Beet armyworm eggs were obtained from the rearing facilities at the USDA-IBPMRL, Tifton, GA. Larvae were reared on a pinto-bean artificial diet following the methodology described by King and Leppla (1984). Insects were kept in a biological incubator with a 14:10 L:D cycle maintained at 25 °C. Third instar larvae were used in all experiments.

Fungal Culture

Sclerotium rolfsii (strain 80) was grown on potato dextrose agar (PDA) petri plates from original cultures provided by Dr. Tim Brenneman (Coastal Plain Experiment Station, University of Georgia, Tifton, GA). Sclerotia from these cultures were harvested, allowed to air dry, and then kept in a dry, dark drawer in the laboratory. Subsequent cultures were started, under sterile conditions, in our laboratory by placing 2-3 of the harvested sclerotia in the center of the PDA media plates. Culture plates were kept in a biological incubator with a 14:10 L:D cycle and maintained at 25°C and 60-70%

RH°. To inoculate the experimental plants, fungal culture plugs were cut out of the agar plate with a # 2 cork borer (5 mm diam).

BAW Feeding on *S. rolfsii*-Infected Peanuts

The feeding preference of BAW larvae on old and young leaves of healthy and fungus-damaged plants was evaluated to determine whether insects were deterred from feeding on tissues of the *S. rolfsii*-infected plants. Peanut plants were infected with the fungus by distributing four culture plugs along the first three inter-nodal spaces of the main stem. The plugs were positioned so the fungus was in direct contact with the stem. Fungal plugs were pressed against the stem so they remained in place. Each plant was then individually covered with a 3.78-L plastic storage bag (Ziploc DowBrands L. P., Indianapolis, IN) to provide adequate humidity and temperature conditions for fungal growth and colonization of the plant's stem. The plants were incubated for 3 d, after which time lesions of approximately 1-cm long could be observed at the point of fungal contact with the stem. After this incubation period, bags were removed from the plants 24 h before being used for the experiment. *S. rolfsii* is a non-systemic pathogen and only the stems of the plants were in contact with the fungus and therefore, the leaves used for the experiments were not infected.

The second-oldest (old) or newest (young) fully expanded tetrafoliate leaves on the mainstem of an infected plant were paired with their counterparts from a healthy plant by confining them within petri dish clip-cages (Fig 2-1). The leaves (four leaflets each), still attached to each of the plants, were placed side by side within a clip-cage (Alborn et al. 1996, Fig 2-1) so the caterpillars had equal access to them. Insects were deprived of food for 6 h before the start of the experiment to ensure immediate feeding on the plant

tissues. Generally, young peanut leaves were larger than old leaves, so six third instar larvae were confined with the young leaves and three larvae of the same stage were confined to the old leaves. Caterpillars were removed from the leaves after 24 h. Leaves exposed to the feeding were carefully labeled, removed from the plants, brought into the laboratory, and photocopied to estimate feeding damage. Leaf images were scanned and imported into an imaging software program (ImagePC beta version 1, Scion Corporation, Frederick, MD) to estimate leaf area eaten and leaf area remaining. These measurements were used to calculate the leaf area consumed by the insects on each of the treatments. Six replicates

of this experiment were set up at one time in the greenhouse, under conditions described above. This experiment was repeated twice more for a total of 18 replicates.

Collections of Volatile Compounds from Fungus and BAW-Damaged Peanuts

In this experiment plant treatments consisted of: a) control (uninfected/undamaged), b) BAW-damaged, c) fungus-infected and, d) fungus-infected plus BAW damage. Plants were inoculated with the fungus as described in the first experiment. BAW-damaged plants were exposed to feeding by six third instar larvae within the volatile collection chambers 3 d after pathogen inoculation and 12 h before the start of the first sampling period.

The aerial portion of the plant was contained within the glass sleeves of guillotine type volatile collection chambers (Fig 2-2) (Analytical Research Systems Inc., Micanopy, FL) that rested on Teflon bases, with an opening that closed around the plant stem (Fig 2-2) (Röse et al. 1996). Purified air was pumped in at the top of the chamber at a rate of 5 L min^{-1}. Air within each of the chambers was sampled daily, at a rate of 1 L min^{-1}, for 4 d

in three consecutive periods: 1) 6:00 am-12:00 pm, 2) 12:00 pm-6:00 pm, and 3) 6:00 pm-6:00 am. Compounds emitted were collected at the downwind end of the chambers in adsorbent traps (Fig 2-2) containing 25 mg Super Q adsorbant (800-100 mesh) (Alltech, Deerfield, IL). All volatiles collections were conducted in the greenhouse where plants were grown. The experiment was set up in duplicate and repeated twice more on different days for a total of six replicates.

Collection of Volatile Compounds from Fungal Cultures

To determine whether any of the volatile compounds collected from infected plants were also produced by the fungus directly, we collected and analyzed volatiles from culture plugs of the white mold at different stages of development. Five 1-cm diameter plugs were made from *S. rolfsii* culture plates at 3, 5, 8, and 16 d after inoculation. Volatiles emitted from control, non inoculated PDA plates, were also collected and analyzed. Plugs from each treatment were separately placed in Pyrex glass odor collection chambers and volatiles were collected using a push-pull system previously described by Turlings et al. (1991). Volatiles were adsorbed by the Super Q traps described previously. Approximately 300 mL/min of purified and humidified air was passed over the culture plugs and through the collection traps for a 2-h period. Air samples were obtained from plugs made from three different culture plates for each of the developmental times tested.

Sample Extraction and Analysis

Compounds from individual traps in the volatile collection experiments were eluted with 170 µL dichloromethane (GC/GC-MS Solvent, B&J, Allied Signal, Inc, MI), and then 400 ng each of *n*-octane and nonyl acetate were added to each eluted sample as

internal standards. The samples were analyzed by gas chromatography with flame ionization detection (HP5890 Gas Chromatograph, HP7673 auto sampler, Hewlett Packard, Palo Alto, CA) equipped with a 15-m × 0.25 mm ID, 0.25-μm film thickness DB-1 capillary column (Quadrex, New Haven, CT). The splitless mode injector system was set at 220°C, the column oven was held at 40°C for 1 min after injection and then programmed at 14°C min^{-1} to 180°C. The carrier gas used was helium at a flow average velocity of 19 cm s^{-1}.

For identification of compounds, selected samples were analyzed via GC/MS (HP 6890 Gas Chromatograph equipped with 30 m × 0.25 mm ID, 0.25-μm film thickness HP-5 capillary column, interfaced to a 5973 Mass Selective Detector, Hewlett Packard, Palo Alto, CA) in both electron impact and chemical ionization modes. The column was held at 40°C for 1 min after injection and then programmed at 10°C min^{-1} to 180°C. The carrier gas used was helium at a flow average velocity of 30 cm s^{-1}. Isobutane gas was used as the reagent gas for chemical ionization, and the ion source temperature was set at 250°C. Individual compounds were identified by comparing their retention times to those of commercially obtained authentic samples and by comparing their mass spectra against those available in a database from the Environmental Protection Agency/ National Institute of Standards and Technology.

Effect of Selected Volatile Compounds from *S. rolfsii*-Infected Peanut on Radial Growth of Fungal Cultures

The effect of vapors from synthetic forms of 3-octanone, (Z)-3-hexenyl acetate, linalool and methyl salicylate (Sigma-Aldrich Chemical Company, St. Louis, MO) on the radial growth of *S. rolfsii* on PDA culture plates was evaluated. These compounds were

selected because they were present in the blend of compounds emitted by infected peanut plants and because they were commercially available. Three levels of the compounds, 3, 10, and 30 μL, were diluted in 100 μL hexane (GC/GC-MS Solvent, B&J, Allied Signal, Inc, MI) and loaded on to 5 ×11 mm sleeve stoppers made of natural red rubber (Wheaton, Millville, NJ). The loaded stoppers were allowed to equilibrate for 24 h before using them for the experiments. The rubber stoppers were attached to the cover of the petri dish by means of double-sided Scotch tape to prevent direct contact and contamination of the growth media. Two *S. rolfsii* sclerotia were placed in the center of the bottom part of the plate containing the PDA media; thus, the fungus was exposed only to the vapors of the compounds emitted from the rubber stoppers for the duration of the experiment. Rubber stoppers loaded with 100 μL hexane were used in the control plates. The radial growth of the fungal cultures under each of the treatments was measured and recorded daily for a total of 4 days. This experiment was repeated on different days for a total of six replicates.

Statistical Analyses

Data for BAW feeding preference were analyzed by paired *t*-test (Proc MEANS, SAS Institute, 1996). Data for volatile collections and the effect of volatiles on fungal radial growth were analyzed using ANOVA (Proc GLM, SAS Institute, 1996). Significant ANOVAs were followed by Tukey's mean separation test.

Results

BAW Feeding on *S. rolfsii*-Infected Peanuts

BAW feeding was not negatively affected by infection of *S. rolfsii* on peanut plants. To the contrary, larvae consumed significantly more of the young and old leaves

from fungus-infected plants than from healthy, non infected plants (Figure 2-3). Fungal infection resulted in a 1.4-and 6.4-fold increase in BAW leaf area consumption for old and young leaves, respectively.

Collection of Volatile Compounds from Fungus- and BAW-Damaged Peanuts

Uninfected/uninfested control plants released small amounts of volatiles compared to the other treatments. Healthy and fungus-infected peanut plants exposed to BAW-feeding started releasing volatiles within 24 h. However, plants exposed to damage by *S. rolfsii* alone did not emit volatiles until the third day of collection. Volatile emission in all plants showed a diurnal pattern with peak release during period 2 (12:00-6:00 pm). Thus, data presented for this experiment are from the 12:00-6:00 pm period of the third day of collection.

Non-inoculated control plants (Fig 2-4A) released relatively small amounts of volatiles, such as myrcene, β-ocimene, linalool, and (*E*)-4,8 dimethyl-1,3,7-nonatriene compared to those released by white mold-infected or BAW-damaged peanut plants. Plants infected with the white mold fungus alone released (*Z*)-3-hexenyl acetate, linalool and relatively large amounts of (*E*)-4, 8-dimethyl-1,3,7-nonatriene (Fig 2-4B), which were also present in emissions from BAW-damaged plants. Additionally, the compounds methyl salicylate and 3-octanone were only present in volatile emissions from plants that were infected by the fungus (Fig 2-4B) and not in those of plants damaged by BAW alone (Figs 2-4C). Non-infected peanut plants exposed to feeding by BAW released large amounts of lipoxygenase products, monoterpenes, indole, and sesquiterpenes (Fig 2-4C). The emission of volatiles from peanuts in response to BAW-damage was not negatively affected by infection of white-mold fungus on the plant. In fact, white mold infected

peanuts damaged by BAW (Fig 2-4D) released all the volatiles typical of a healthy plant damaged by BAW. Furthermore, the amounts of some released compounds, such as (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, myrcene, and (*E*)-4, 8-dimethyl-1,3,7-nonatriene, were higher than those emitted from non infected plants in response to BAW feeding (Figs 2-4C). In addition, headspace collections from white mold/BAW-damaged plants also contained the unique volatiles produced by plants in response to white mold infection alone (Fig 2-4B).

Collection of Volatile Compounds from Fungal Cultures

Volatile profiles from *S. rolfsii* cultures showed the presence of one dominating volatile compound which was identified as 3-octanone. Amounts of 3-octanone produced by the fungal culture plugs increased with age up until day 8, and ceased almost completely by day 16. Mean (SD) ng/h of 3-octanone produced were 0.9 (0.4), 1.8 (0.3), 4.3 (0.7), and 0.1 (0.2) at days 3, 5, 8, and 16, respectively. This compound was recovered only from culture plates containing the fungus and not from non-inoculated control PDA plates.

Effect of Selected Volatile Compounds from S. *rolfsii* -Infected Peanut on Radial Growth of Fungal Cultures

The growth rate of the fungus was significantly reduced by (Z)-3-hexenyl acetate, linalool and methyl salicylate volatiles released from septa treated with 10μL Additionally, volatiles from 30μL of linalool or methyl salicylate on a septum completely inhibited sclerotial germination and fungal growth (Fig 2-5). The *S. rolfsii*-produced compound, 3-octanone, did not prevent sclerotial germination and significantly reduced the growth of the fungus only at the highest dose of 30 μL tested.

Discussion

Plant release of volatile compounds in response to attack by herbivores, and the role of such compounds in attracting parasitoids of the herbivores, have been studied extensively in recent years (Turlings et al. 1991a, b, 1993, McCall et al. 1994, Loughrin et al. 1995, Röse et al. 1996, 1998, Pare & Tumlinson 1997). In contrast, the emission of volatiles in response to pathogen invasion has been the subject of a limited number of studies (Croft et al. 1993, Doughty et al. 1996, Shualev et al. 1997, Chaudry et al. 1998, Buonaurio & Servili 1999). All of these studies have examined the induction of plant volatile emission by either herbivore damage or pathogen infection on different plant systems or using excised plant parts. Thus, there is no clear knowledge of whether the regulation of volatile production in response to these organisms is affected in any way by the simultaneous attack of herbivores and phytopathogens on the same plant.

Data obtained from this study confirm that peanut plants release volatile compounds in response to attack by the white mold fungus. Furthermore, I present conclusive evidence that the volatile profile emitted by these plants differs qualitatively and quantitatively from profiles emitted from healthy plants and from those emitted in response to BAW damage. Additionally, previous infection of the plant by the white mold fungus does not interfere with the emission of volatiles by the diseased plant in response to BAW attack; rather it seems to induce release of some compounds in relatively higher quantities.

The significant quantitative difference in the volatile profile of peanut plants attacked by either the fungus or the insect, added to the fact that emission of compounds was not suppressed when both organisms were simultaneously attacking the plant,

provides a clear indication that the activation and regulation of plant biosynthetic pathways is dependent upon the type of threat perceived by the plant. Four major biosynthetic pathways are believed to be involved in the production of plant volatiles in response to damage by lepidopterous larvae (Paré & Tumlinson 1997). The shikimic/ tryptophan, mevalonate, Rohmer, and lipoxygenase pathways are all believed to be involved in the production of the different classes of volatile compounds released by plants under attack by insect herbivores (Paré & Tumlinson 1997). The lipoxygenase (LOX) pathway, via jasmonate production, has been directly linked to direct plant defense responses to wounding and damage by herbivorous insects (Ryan 1990, Farmer & Ryan 1990, Farmer et al. 1992). Jasmonic acid and methyl jasmonate have also been found to induce volatile emissions similar to those resulting from herbivory in many plants (Boland et al. 1995, Dicke et al. 1999). Thus, it has been suggested that JA and its methyl ester mediate volatile production. In my study, the emission of volatiles from peanut plants previously infected with *S. rolfsii* indicates that, in this case, unlike direct defenses in other plants, volatile production is not compromised by pathogen infection. The latter may be an indication that peanut production of volatile compounds in response to insect and pathogen attack is not jasmonate-dependent. However, the precise combination and regulation of pathways activated in response to an individual or combination of stressors may vary widely between plant species. Therefore, the role of JA and SA on the production and release of induced volatiles by plants in response to insect and pathogen attack were investigated further in Chapter 5. The dynamics of volatile emission in response to insect herbivores, phytopathogens, and their combined effect upon other plant species also merit additional attention.

Volatile compounds have been shown to affect pathogens in different ways. For example, the germination and growth of white mold are stimulated by the release of methanol and other volatile compounds from moist peanut hay (Shokes et al. 1996). On the other hand, volatiles from ground-up healthy corn kernels resistant to *Aspergillus flavus*, have been found to inhibit the growth and aflatoxin production in colonies of this pathogen (Zeringue et al. 1996). In cotton, however, the lipoxygenase-derived volatile (*E*)-2-hexenal inhibited, while α-and β-pinene stimulated the growth of this fungus (Zeringue and McCormick 1989, 1990). Thus, the negative effect of (*Z*)-3-hexenyl acetate, linalool, and methyl salicylate volatiles on the growth of *S. rolfsii* in my laboratory cultures suggests that the production of these volatiles by the plant acts as a direct defense by slowing the growth of the fungus and preventing additional sclerotial germination. However, since my attempts to quantify the headspace concentration of the compounds used in my study failed, further experiments need to be conducted to confirm that the amounts used here are comparable to those produced by an infected plant, and that in fact the growth of the fungus is hindered under natural conditions.

In the dual-choice feeding experiments, BAW preferentially fed on leaves from white mold-infected peanut plants. BAW and corn earworm, *Helicoverpa zea*, have been reported to feed more upon tomato plants that had been previously treated with benzothiadiazole-carbothionic acid S-methyl ester (BTH), an elicitor of systemic acquired resistance via the salicylic acid pathway (Stout at al. 1999). Tomato plants treated with BTH were found to have compromised direct defenses, based on expression of genes encoding for proteinase inhibitors (Fidantsef et al. 1999). In my study, the presence of methyl salicylate in headspace samples of white mold infected plants indicates activity of

the salicylic acid pathway in peanuts in response to *S. rolfsii* infection. Thus, the feeding preference observed in BAW towards leaves from white mold infected peanut plants may be caused by a reduction in direct defenses of the plant due to fungal infection. Alternatively, the feeding preference of BAW for leaves from fungus-infected plants could be due to changes in the nutritional quality of the tissues caused by the pathogen infection. Different pathogen species have been found to either up -or down-regulate accumulation of photoassimilates (reviewed in Hatcher 1995). Pathogen infection influences concentrations of compounds such as nitrogen, non-structural carbohydrates, starch, protein, free amino acids, and amino acid composition, in their respective plant hosts (reviewed in Hatcher 1995). Although most of the studies included in that review evaluated the effect of leaf-infecting pathogens on the suitability of plant tissue to insect herbivores, it is not inconceivable that a stem-infecting pathogen such as *S. rolfsii* can have similar consequences upon its hosts. This is an aspect that was investigated in Chapter 3.

To my knowledge, this is the first *in planta* study in which the production of volatiles by a single host system in response to both insect herbivores and pathogens has been evaluated simultaneously. This is also the first time that the effect of previous pathogen infection on the production of plant volatiles in response to insect damage has been studied. The study of plant volatile defenses may improve our understanding of plant resistance mechanisms to disease and insect herbivores. The identification of specific pathogen- and herbivore-induced plant volatiles will greatly contribute to the development, improvement and implementation of host-plant resistance and other control methods for insect and pathogen pests.

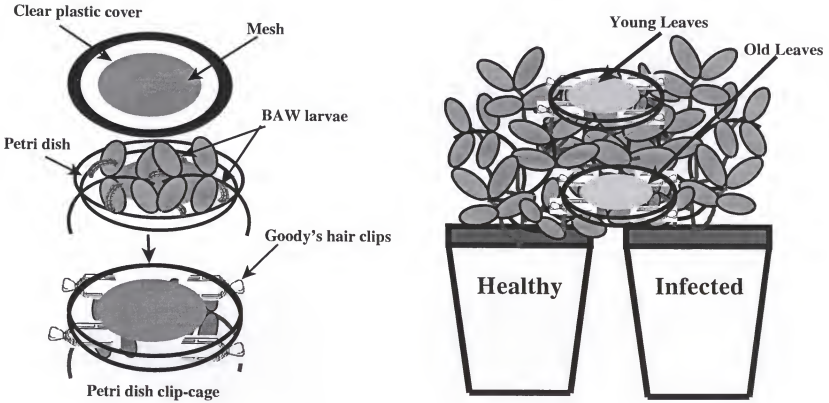


Figure 2-1. Petri dish clip-cages used in the dual-choice feeding experiment to test BAW feeding preference on Leaves from healthy and fungus-infected peanut plants

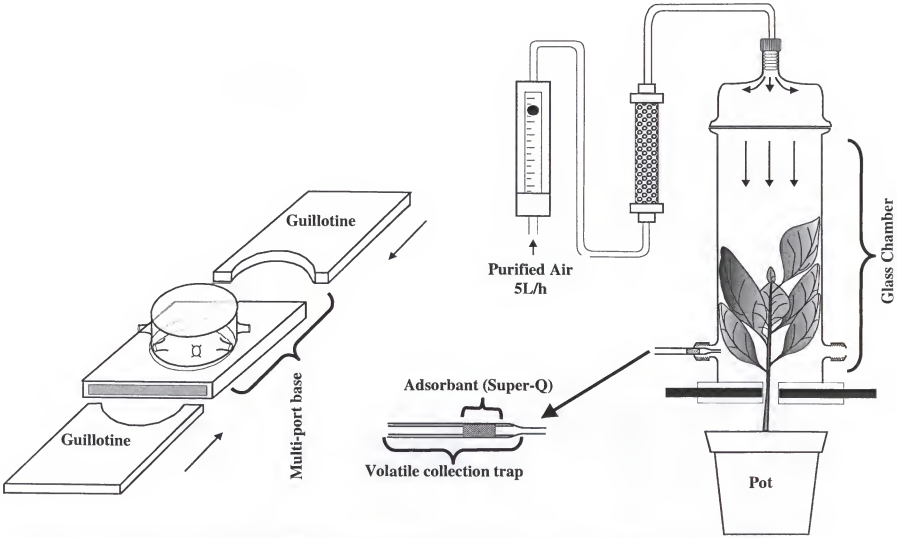


Figure 2-2. Volatile collection system used to sample volatile compounds emissions from healthy, BAW-damaged, pathogen-infected, and pathogen + BAW-damaged plants

Figure 2-3. Mean leaf area consumed by third instar *Spodoptera exigua* larvae in paired-choice tests with healthy (dark bars) and white mold-infected (white bars) plants. A) Old leaves and B) young leaves. Error bars denote 1 SE and * denotes significant differences (paired *t*-test, $P \leq 0.05$).

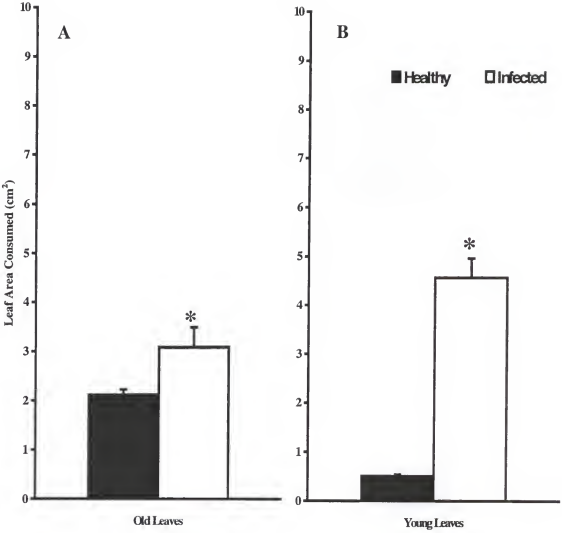

A
B
Healthy
Infected
Leaf Area Consumed (cm²)
0
1
2
3
4
5
6
7
8
9
10
*
*
Old Leaves
Young Leaves

Figure 2-4. Mean volatile emissions from peanut plants A) Uninfected/undamaged, B) White-mold infected, C) BAW-damaged, and D) White mold infected + BAW damaged plants. Compounds: 1) *(E)*-2-hexenal, 2) *(Z)*-3-hexen-1-ol, 3) α - pinene, 4) β- pinene, 5) 1-octen-3-ol, 6) 3-octanone, 7) *(Z)*-3-hexenyl acetate, 8) myrcene, 9) eucalyptol, 10) limonene, 11) β-ocimene, 12) linalool, 13) *(E)*-4, 8-dimethyl-1,3,7-nonatriene, 14) *(Z)*-3-hexenyl isobutyrate, 15) methyl salicylate, 16) *E*-2-hexenyl butyrate, 17) *Z*-3-hexenyl butyrate, 18) indole, 19) *(Z)*-jasmone, 20) β- caryophyllene, 21) 2, 6,-dimethyl-6-(4-methyl-3-pentenyl) bicyclo [3.1.1] hept-2-ene**, 22) α-farnesene, 23) α-humulene, 24) germacrene-D**, 25) β-farnesene, 26) naphthalene**, 27) nerolidol, 28) (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Day 3, period 2 (12:00 pm-6:00 pm). Error bars are equivalent to 1 SE. **No synthetic standards available, identification is based on National Institute of Standards and Technology library spectral match only.

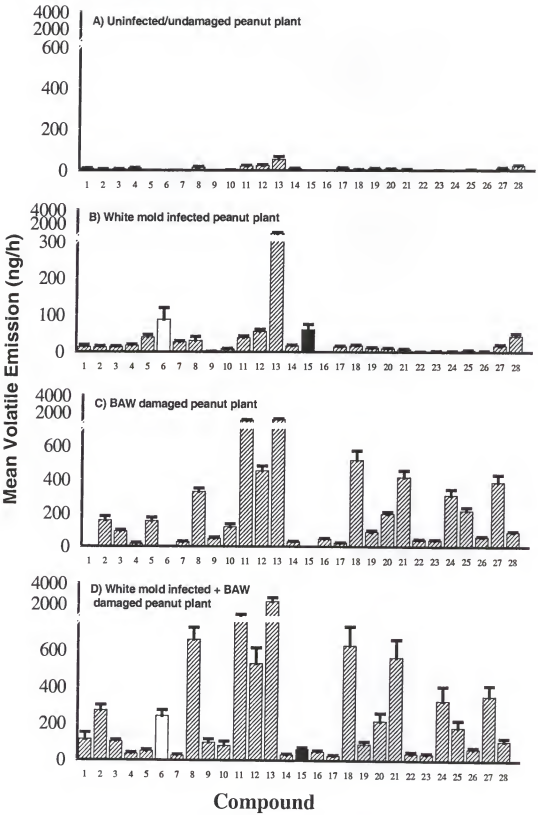
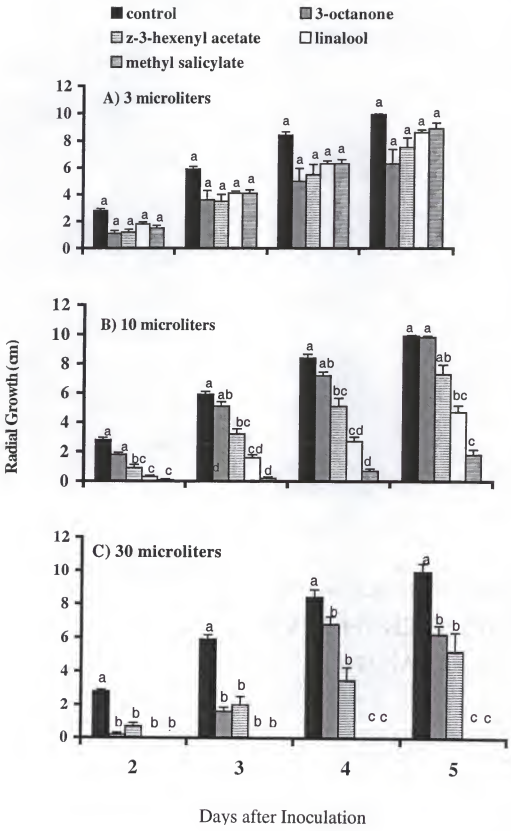

A) Uninfected/undamaged peanut plant
B) White mold infected peanut plant
C) BAW damaged peanut plant
D) White mold infected + BAW damaged peanut plant
Mean Volatile Emission (ng/h)
Compound

Figure 2-5. Mean radial growth of *S. rolfsii* cultures exposed to volatile vapors from 3-octanone, (*Z*)-3-hexenyl acetate, linalool and methyl salicylate A) 3 µl, B) 10 µl, and C) 30 µl of the synthetic forms of the compounds. Bars within days headed with the same letter are not significantly different (Tukey's mean separation test, $P \geq 0.05$). Error bars indicate 1 SE.


control
3-octanone
z-3-hexenyl acetate
linalool
methyl salicylate
A) 3 microliters
B) 10 microliters
C) 30 microliters
Radial Growth (cm)
Days after Inoculation
2
3
4
5

CHAPTER 3
FUNGUS-INDUCED BIOCHEMICAL CHANGES IN PEANUT PLANTS AND THEIR EFFECTS ON DEVELOPMENT OF BEET ARMYWORM, *SPODOPTERA EXIGUA* HÜBNER (LEPIDOPTERA: NOCTUIDAE) LARVAE

Introduction

Herbivorous insects and plant diseases are serious problems in agriculture because they reduce yield and quality of crops. The study of physiological and chemical changes induced in plants by the attack of insects and pathogens has captured the interest of scientists in the areas of plant pathology and entomology, independently. The primary focus of the resulting studies has been to develop potential plant immunization methods as a means to prevent pest outbreaks (Yedidia et al. 1999, Lucas 1999). Systemic acquired resistance (SAR) is a non-specific resistance induced throughout the plant by local infection with a variety of pathogens (Kuc 1982, Dean and Kuc 1985). Recent studies have provided evidence for the key role of salicylic acid (SA) in inducing SAR (Delaney et al. 1994, 1995). Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) is a synthetic SA analog capable of inducing SAR. BTH also induces disease resistance in a number of plant species and this SAR is not due to an accumulation of SA (Friedrich et al. 1996).

Different plant defense pathways are generally induced in response to insect herbivores and pathogen attack. Jasmonic acid (JA) induces expression of defensive compounds such as proteinase inhibitors and polyphenol oxidases, which provide plant resistance against insects (Ryan 1990, Farmer and Ryan 1990, Farmer et al. 1992, Thaler et al. 1996, 2001). Salicylic acid (SA), on the other hand, is involved in the production

of pathogenesis-related proteins and phytoalexins in response to pathogens (Delaney et al. 1994, Narusaka et al. 1999, Murphy et al. 2000). While previous exposure to a pathogen can provide plant resistance to future attacks by the same pathogen species, the induced changes can also have interspecific consequences. Pathogen infection can alter host plant biochemistry in such a way that the feeding preference and performance of phytophagous arthropods may be positively or negatively affected. For example, Karban et al. (1987) reported that the development of spider mites, *Tetranychus urticae*, was negatively affected when they fed on cotton plants that had been previously inoculated with the fungus *Verticillium dahliae*. In contrast, beet armyworm (BAW), *Spodoptera exigua*, larvae preferentially fed and consumed larger quantities of leaves from white mold, *Sclerotium rolfsii*, infected peanut plants (Cardoza et al. 2002). Tomato plants that had been previously treated with BTH, were fed on to a greater extent by BAW and corn earworm, *Helicoverpa zea*, than untreated controls (Stout at al.1999, Thaler et al. 1999). In a study of proteinase inhibitor gene expression, tomato plants treated with BTH were determined to have compromised direct defenses against herbivores (Thaler et al. 1999, Fidantsef et al. 1999). Thus, the finding that BAW preferred leaves from white mold infected peanut plants (Cardoza et al. 2002) could have been caused by compromised direct defenses of the plant due to fungal infection. On the other hand, pathogens have been found to affect the levels of compounds such as sugars, starch, and proteins in their host plants (reviewed in Hatcher 1995). Major nutrients such as carbohydrates and proteins are listed as some of the most important phagostimulants for phytophagous insects (Matsuda 1988), whereas, secondary plant defensive compounds such as tannins (phenolic) and protease inhibitors can have the opposite effect (Slansky & Scriber 1985). Therefore, it is possible that the feeding preference of BAW for leaves from fungus-infected plants could have been influenced by changes in secondary metabolite content and/or the nutritional quality of the plant tissues.

In the present study, I examined the effect of *S. rolfsii* infection on the levels of primary (nutrients) and secondary (defense substances) metabolites, and on the levels of plant defense signaling molecules in peanut plants. Experiments were also conducted to evaluate the performance of BAW larvae, from third instar to pupation, on either healthy or white mold infected peanut plants. The levels of nutrients, such as sugars, starch, and proteins, and of secondary defense substances such as soluble phenolics, and proteinase inhibitors in leaves of healthy and infected plants were compared. The latter analyses were conducted to determine a possible correlation between the levels of these compounds and the performance of the insects on these plants. Levels of the defense signaling molecules JA and SA in plants under individual and simultaneous pathogen and BAW attack were also determined.

Materials and Methods

Plant and Insect Material

'Georgia Green' peanut, *Arachis hypogeae*, seeds were sown in pairs in 3.78-L pots (16-cm diam) containing a 1:1 (vol:vol) mixture of commercially available filter sand and Metromix 300 (Scotts-Sierra Horticultural Company, Marysville, OH). Plants were grown in an insect-free greenhouse with natural light, under Florida summer conditions (14L:10D light cycle). The greenhouse temperature was kept between 25-30°C. After emergence, seedlings were thinned to one individual per pot. Each plant received 100 mL of a 3.38 g/L liquid fertilizer solution (20-20-20 [N-P-K] Peters, W. R. Grace, Fogelsville, PA) every two weeks starting one week after emergence. At the beginning of all experiments, plants were five-weeks old and had six fully developed leaves on the main stem and three fully developed leaves on each of two secondary branches. Beet armyworm eggs were obtained from the rearing facilities at the USDA-IBPMRL, Tifton, GA. Larvae were reared on a pinto bean-based artificial diet following the methodology described by King and Leppla (1984). Insects were kept in a biological

incubator with a 14:10 L:D cycle maintained at 25 °C. Newly-molted third instar larvae were used in all experiments.

Fungal Culture

Sclerotium rolfsii (strain 80) was grown on potato dextrose agar (PDA) petri plates. Fresh cultures were started, under sterile conditions, by placing sclerotia harvested from the original culture in the center of PDA media plates. Culture plates were kept in a biological incubator with a 14:10 L:D cycle and maintained at 25°C and 60-70% RH° for 3 d. To inoculate the experimental plants, fungal culture plugs (5 mm diam) were cut out of the agar plate with a # 2 cork borer.

Sclerotium rolfsii Infection on Peanuts

Peanut plants were infected with *S. rolfsii* by distributing four culture plugs along the main stem. The plugs were pressed against the stem so they remained in place and so the fungus was in direct contact with the stem. Each plant was then individually covered with a 3.78-L plastic storage bag (Ziploc DowBrands L. P., Indianapolis, IN) to provide adequate humidity and temperature conditions for fungal growth and colonization of the plant's stem. Non-inoculated control plants were similarly covered with the plastic bags. The plants were incubated for 3 d in the greenhouse. After this period, lesions of approximately 1-cm long could be observed at the point of fungal contact with the stem. Bags were removed from the plants 24 h before being used for the experiment. *S. rolfsii* is a non-systemic pathogen and only the stems of the plants were in contact with the fungus; therefore, the leaves that were consumed by the caterpillars were not directly infected by the fungus.

BAW Performance on Healthy and Fungus-Infected Peanut

Healthy and fungus-infected plants were individually placed within 46 × 46 × 46 cm plexiglass cages into which 6 newly-molted third instar BAW were introduced in a 1

oz plastic cup and placed on top of the pot at the base of the plant's stem. Cages were kept in the greenhouse throughout the experiment. One plant provided more than enough food for all insects to develop until pupation. Insects were observed daily and were removed from the plants when they reached the wandering stage and no more feeding activity was observed. All insects from each of the treatments were collectively placed into a petri plate carefully labeled to indicate plant treatment and replicate number and kept in an incubator, under the conditions described above, until pupation. At that time, the time of pupation, number of surviving pupae and their weights were recorded. Four replicates of this experiment were set up at one time in the greenhouse and the experiment was repeated two more times to obtain a total of 12 replicates.

Plant Samples

Plant samples consisted of 8 tetrafoliate leaves removed from the main-stem of plants that were treated as follows: a) healthy (no fungal infection, no insect damage), b) exposed to feeding by six 3rd instar BAW for 24 h, c) fungus infected 4 d before harvesting, d) fungus-infected 4 d before and exposed to feeding by six 3rd instar BAW for 24 h before tissue was harvested. Insects had free access to all plant leaves during this time. After removing the insects from the plants, all the leaves (damaged + undamaged) of 6 plants from each of the above treatments were finely ground in liquid N_2 with a mortar and pestle immediately after removal from the plant. All leaf samples were kept in a -70 °C freezer until needed for the analyses.

Carbohydrates Analysis

Five hundred mg (fresh weight) of the ground leaf samples were combined with 2 mL of 80% acetone: H_2O and then incubated in a 75 °C water bath for 10 min. Then, the samples were centrifuged at 5000 × g for 10 min, the supernatants were transferred to new tubes and the pellets were re-extracted with 2 mL of 80% acetone: H_2O and centrifugation was repeated. The supernatants were combined and then evaporated to

dryness in a Savant SC210A speed-vac (Savant Instruments, Inc. Holbrook, NY). The residue was re-suspended in 1 mL of purified H_2O and this was used to estimate total soluble sugars. Total soluble sugars and glucose from starch digestion were determined by the phenol-sulphuric acid method (Dubois et al. 1956). The remaining insoluble residue was dried overnight in a 70 °C oven. The following day, the residues were gelled by adding 0.20 mL 95% ethanol and 4 mL of 50mM acetate buffer (pH=4.5). The gelled residues were placed in boiling water bath for 15 min and then cooled by placing in an ice bath before adding 0.1 mL of heat stable α-amylase solution (68300 U/mL) (Sigma Chemical Co., St. Louis MO). Samples were incubated overnight at 50 °C and then centrifuged at 5000 × g for 12 min. The supernatant was then used to estimate the amount of glucose cleaved from starch using the phenol-sulphuric acid method .

Protein Analysis

For soluble protein quantification, 50 mg of each of the leaf samples were homogenized in 500 µl of chilled buffer-A, consisting of 0.05 M $NaPO_4$ at pH 7.5 (Sigma Co., St. Louis, MO, USA). The homogenate was centrifuged at 14000 × g for 15 min and the supernatant containing soluble proteins was transferred to a new micro-centrifuge tube. The pellet was washed and centrifuged, as above, three times using 1mL of buffer-A each time and discarding the supernatant. For quantification of insoluble protein, the pellet was re-suspended by vortex mixing in 500 µL of buffer-B, consisting of 62.5 mM Tris-HCl pH 6.8, 2% SDS (w:v) and 10% glycerol (v:v). Following centrifugation at 14000 × g for 15 min, the supernatant containing the buffer-insoluble proteins was transferred to a new microcentrifuge tube. Protein quantity was determined with the Bio-Rad DC Protein Assay (Alam 1992) using bovine serum albumin fraction V as a standard.

Proteinase Inhibitors Analysis

Trypsin proteinase inhibitor activity was determined following the method of Hummel (1958), where *N*-α-benzoyl-DL-arginine p-nitroanilide (BAPNA) is used as a colorimetric substrate. Amount of trypsin proteinase inhibitors is expressed in μg/g fresh weight estimated using commercially available p-nitroaniline (Sigma Chemical Co., St. Louis, MO) as a standard. Cysteine proteinase inhibitor activity was determined using the papain assay based on Koiwa et al. (1998). In this case, *N*-α-benzoyl-DL-arginine-β-naphthylamide (BANA) was used as the colorimetric substrate. In both cases absorbance was read with a spectrophotometer (Spectro 22, Labomed, Culver City, CA) set at 430 nm for the trypsin and at 540 nm for the cysteine proteinase inhibitors. Cysteine inhibitory activity is expressed as the percentage decrease in absorbance relative to the reaction with no plant extract.

Soluble Phenolic Analysis

For extraction of soluble phenolics, 500 mg of the samples were extracted with 10 mL of 1% HCl in methanol, vortexed for 20 min and then centrifuged at 1000 × g for 10 min. The amount of total soluble phenolics in each of the samples was determined using the Folin-Denis method (Swaine and Goldstein 1964). This procedure determines total free phenolics based on the reduction of phosphomolybdic acid by phenols. Absorbance was read in a spectrophotometer (Spectro 22, Labomed, Culver City, CA) set at 760 nm wavelength. Commercially available tannic acid (Sigma Chemical Co., St. Louis, MO) was used as a standard.

Jasmonic Acid and Salicylic Acid Analysis

The extraction and quantification of JA was modified from Weber et al. (1997). Leaf tissue samples of approximately 1 g were extracted in 3.5 mL MeOH with 500 ng of the internal standard dihydrojasmonic acid (dhJA) (Sigma Chemical Co., St Louis, MO). After 30 min in a sonicating bath, each sample was mixed with 1.5 mL of purified H_2O,

and centrifuged at 3000 × g for 5 min. The resulting supernatant was saved, adjusted to pH 8.5 with aqueous 1M NH_4OH and kept on ice. Solid phase extraction (SPE) cartridges (Reverse Phase C18, 12 mL, Mallinckrodt Baker, Griesheim, Germany) were washed with 8 mL each of 100% MeOH, followed by 70% MeOH. Each sample was passed through the SPE cartridge followed by 7 mL of 75% MeOH. All eluate was collected, adjusted to pH 3.5 with 10% formic acid, and the volume was raised to a total of 50 mL with H_2O. The SPE cartridges were cleaned and conditioned for re-use with 5 mL each of 0.8% formic acid in MeOH, 100% MeOH, diethylether, 100% MeOH, and finally 10 mL H_2O. The samples were then re-loaded on the cartridges and washed with 7 mL each of 85:15 H_2O: EtOH and H_2O. All water was removed from the columns by applying vacuum, then the oxylipin fraction was eluted with 10 mL diethylether, and the eluate was transferred to a 2 mL reaction vial where it was dried under N_2. Methanolysis was performed by incubating with 30 µl of a 1:2 (v:v) HCl:MeOH mixture for 12 h at 30 °C. The HCl:MeOH was then completely removed under a stream of N_2 gas and each sample was brought up to 75 µl in CH_2Cl_2. Samples were analyzed by gas chromatography-mass spectrometry (GC-MS) on a Hewlett-Packard (HP) 6890 GC (He carrier gas; 0.7 mL min^{-1}; splitless injector 240 °C, injection volume 2 µl) with a HP-5MS column (5% phenyl methyl siloxane , 30m x 250mm i.d. x 0.25 mm film thickness) with the temperature programmed from 40 °C (1 min hold) at 10 °C min^{-1} to 240 °C (hold for 15 min). The GC was coupled to a HP 5973 quadrapole-type mass selective detector with transfer line, source, and quadrapole temperatures of 230 °C, 230 °C and 150 °C, respectively. Chemical ionization with isobutane as the reaction gas generated predominantly M+1 parent ions scanned at a range of 60-500 amu.

Salicylic acid levels were measured following a procedure by Uknes et al. (1993). Ground plant tissue (0.5 g) was first extracted with 3 mL of 90% methanol, then re-extracted with 2 mL 100% methanol. The extracts were combined, divided into 2 equal

parts, and carefully dried under vacuum. For free SA determination, samples were re-suspended with 2.5 mL 5% trichloracetic acid (TCA). For the estimation of conjugated SA, samples were brought up in 1 mL of water, acidified to a pH of 1 with HCl and boiled for 30 min to cleave the glycoside conjugates. Separate free and conjugated SA samples were each extracted with 2.5 mL ethyl acetate: cyclopentane: isopropanol (100:99:1), dried down and then re-suspended in 20% methanol. The amount of SA in each of the samples was determined by reverse phase high performance liquid chromatography on a 4.6 × 250 mm 5µm C-18 column (Beckman Ultrasphere, Fullerton, CA) with a Waters 474 scanning fluorescence detector (excitation energy 295 nm, emission energy of 400 nm). Data presented are the total (free + conjugated) SA.

Statistical Analyses

Data for performance of BAW on healthy and fungus-infected peanut were analyzed by *t*-test (Proc MEANS, SAS Institute, 1996). Data on nutritional analysis and on levels of JA and SA of peanut leaves were analyzed using ANOVA (Proc GLM, SAS Institute 1996). Significant ANOVAs were followed by Tukey's mean separation test.

Results

BAW Performance on Healthy and Fungus-Infected Peanut

In a no-choice situation, BAW larvae reared on fungus-infected peanut plants had significantly higher survival to pupation (t=3.37; df=53; P=0.0014) (Fig 3-1A), developed significantly faster, based on number of days from 3rd instar to pupation (t=29.9; df=53; P=0.0001) (Fig 3-1B), and produced significantly heavier pupae (t=2.96; df=53; P=0.0045) (Fig 3-1C) than on their healthy counterparts. Overall, insects reared on fungus-infected peanut plants had approximately 20% higher survival rate, developed more than 4 days faster and were 25% heavier than those reared on healthy plants.

Plant Nutritional Analysis

Plants infected by the fungus had significantly higher soluble sugar content (F=10.6; df=3, 36; P<0.0001) and significantly lower starch content (F=4.8; df=3, 36; P<0.0064) than healthy plants and plants damaged by BAW alone (Table 3-1). Soluble and insoluble protein, and trypsin proteinase inhibitor activity were not significantly different among the treatments tested. However, cysteine proteinase inhibitor activity was higher in plants attacked simultaneously by the fungus and BAW (F=2.68; df=4, 156; P<0.0489) compared to undamaged control plants or those that were fungus-infected or damaged by BAW alone (Table 3-1). Additionally, the concentration of tannins (soluble phenolic) was significantly lower in plants infected with fungus (F=3.5; df=4, 35; P<0.0163).

Jasmonic Acid and Salicylic Acid Analysis

Plants that were infected with the white mold fungus or damaged by BAW had similar levels of JA (Table 3-1). However, plants that were infected by the fungus and then exposed to BAW had the highest detectable levels of JA and these were significantly different from those in all of the other treatments (F=2.63; df=4; P<0.0001) (Table 3-1). JA was not detected in undamaged/uninfected control plants (Table 3-1). Levels of salicylic acid in plants with all treatments, except BAW alone, were not significantly different from each other (Table 3-1). However, the levels of SA in plants damaged by the insects alone were significantly lower than those in the fungus/BAW treated plants (F=3.86; df=4; p<0.001) (Table 3-1).

Discussion

BAW have been reported to prefer feeding on leaves from white mold-infected over those from healthy peanut plants (Cardoza et al. 2002). In the present study, I found that these insects survived better, developed faster, and reached a greater pupal weight when reared on white mold-infected peanut plants. Following nutritional analysis of leaf

tissue, I found that leaves from fungus-infected plants had higher levels of nutrients (sugars) and lower levels of defensive substances (soluble phenolics) than their healthy counterparts, which may have contributed to the observed BAW feeding preference and positive effect on their performance. Some species of grasshoppers prefer feeding and perform better on sunflower leaves infected by a rust fungus (Lewis 1979, 1984). Sucrose has been found to be a strong phagostimulant for grasshoppers and other insects (Cook 1977, Dethier 1982, Bernays & Weiss 1996); thus, BAW feeding preference and enhanced performance may have been due to the higher concentration of soluble sugars I found in the tissue from white mold infected plants. Plant phenolics, commonly referred to as tannins, are believed to reduce food ingestion by herbivores due to their astringent properties (Levin 1971). These compounds can form complexes with proteins in general; thus, they may interfere with absorption of plant protein and may affect the activity of herbivore digestive enzymes (Levin 1971,1976; Felton, et al. 1989, 1992; Haggerman & Butler 1991). Phenolic compounds have also been reported as being induced in plants by fungal infection (Levin 1976) and they have been found to confer resistance to plants against certain fungal pathogens (Walker & Stahmann 1955). Thus it was surprising to find that the level of soluble phenolics in plants infected by the white mold in my study was actually lower than that in control plants and in plants damaged by BAW alone.

Trypsin-like proteinases are the major active digestive proteinases in the guts of *Spodoptera* larvae (Broadway 1988). Plant proteinase inhibitors, especially in the serine class, such as trypsin and chymotrypsin inhibitors, have been reported to slow larval development in BAW and other lepidopteran larvae (Broadway & Duffy 1986, Broadway et al. 1986). The development of BAW was significantly retarded with the addition of as little as 0.045% potato chymotrypsin II and 0.09% w. wt. soybean trypsin inhibitor to a casein-based diet (Broadway & Duffy 1986). Plant proteinase inhibitors have also been found to negatively affect the growth of fungal pathogens (Lorito et al. 1994). Recently, a

trypsin-like proteinase inhibitor with antibiotic properties against the fungus *Sclerotinia sclerotiorum* has been isolated from sunflowers (Giudici et al. 2000). In my study, the level of trypsin-like proteinase inhibitors did not differ among the different treatments, but the level of cysteine proteinase inhibitor activity was significantly higher in plants infected by the white mold fungus. Recent studies suggest that cysteine proteinase inhibitors have a very active role in the defense of plants against pathogens (Giri et al. 1998, Joshi et al. 1999).

The effect of fungal infection on insect feeding preference and their performance on plants varies widely. For example, Kluth et al. (2001) found that when given a choice between healthy and rust-infected thistle, chrysomelid beetles preferred to feed on the healthy plants. Hatcher et al. (1995) found that infection of *Rumex* spp. by rust fungi increased feeding preference by the chrysomelid beetle *Gastrophysa viridula*, but larval performance and adult reproductive capacity were negatively affected. In the same study, rust-infected leaves were found to have lower nitrogen and higher oxalate concentration than healthy leaves, which might account for the negative effects observed in the insects. Moran (1998) found that discs from cucumber, *Cucumis sativus*, leaves infected with *Cladosporium* fungi were preferably fed on by cucumber beetles, *Diabrotica undecimpunctata howardii*. Similarly, other studies have found that plant defensive responses against pathogens have a negative effect on the plant's ability to cope with insect herbivory (Karban et al. 1987, Bostock 1999, Felton et al. 1999, Fidantsef et al. 1999, Stout et al. 1999). On the other hand, there have also been reports suggesting enhanced plant defenses against insects in plants following pathogen inoculation. For example, McIntyre et al. (1981) found that infection of tobacco, *Nicotiana tabacum*, plants by the tobacco mosaic virus increased resistance to green peach aphids, *Myzus persicae*. Direct and indirect interactions between other plant pathogenic fungi and

insects species with either negative, positive or neutral outcomes for the insect are reviewed in Hatcher (1995).

The JA and SA pathways play an important role in plant defense against insect herbivores and pathogens. In the present study, I analyzed leaf tissue from peanut plants that were under individual or simultaneous attack by the white mold fungus and BAW to determine their levels of JA and SA. Peanut plants infected with the white mold fungus, in my study, had levels of JA that were comparable to those induced by BAW damage alone. Additionally, plants that were infected with the fungus and exposed to BAW damage, not only had significantly higher levels of JA than those damaged by either pest alone, but also had higher JA levels than could be explained by their additive effect upon the plant. JA was not detected in my control; it is possible, however, that JA levels in healthy peanut plants fall below the range of sensitivity of the method used in this study. Since SA has been reported to be involved in plant defense responses against pathogens, it was surprising to find that the levels of SA in peanut plants infected by *S. rolfsii* alone did not differ significantly from those of healthy plants. Because the levels of SA in white mold infected peanuts were actually lower than in healthy plants, it is unlikely that the feeding preference of BAW for white mold-infected peanut was due to SA suppression of JA-modulated defenses. Furthermore, plants under simultaneous attack by both pests had the highest levels of both JA and SA. Yet, it is interesting to note that despite the high levels of JA in these plants, the concentrations of trypsin proteinase inhibitors, which would confer resistance to BAW (Broadway and Duffey 1986), were not different from those in all other plant treatments. Plants exposed to damage by BAW alone had the lowest levels of SA compared to all other treatments, so it appears that levels of this compound in peanuts are suppressed below normal levels when healthy plants are damaged by the insects alone.

It is now evident that the role of these two pathways in insect and pathogen defense is not as clear as previously thought. In fact, my data suggest that the JA rather than the SA pathway may be the one responsible for peanut defenses against white mold infection, and that these pathways in peanut are up- or down-regulated depending on the number and type of biotic factors affecting the plant. The JA pathway has been previously reported to confer resistance in plants to other fungal pathogens (Vijayan et al. 1998, Cohen et al. 1993, Schweizer et al. 1993).

Sclerotium rolfsii is an opportunistic necrotrophic fungus, which, under favorable conditions eventually overcomes and kills its hosts. Thus, it is possible that the positive effect of its infection of peanuts on the performance of BAW may change with the amount of initial inoculum and also with the progression of the disease over time. For these reasons, it is important to note that the levels of fungal inocula used in my study were low and only caused a sub-lethal infection of the peanut plants.

Most studies conducted on the induction of plant defenses have focused on the effect of individual pest species; however, under natural field conditions, plants may often be under attack by different insect and pathogen pest species, or combinations thereof, at any given time. Understanding the innate mechanisms of defense in plants against multiple pests is vital for future development of resistance in crops. The data presented here contribute towards a better understanding of the effects of the interactions between insect and pathogen pests on their host plants. This type of information is important for the development of tools to prevent, manage, and control pests in agricultural environments.

Table 3-1 Mean (SE) amounts of selected nutrients and hormones in leaves of healthy (Control), BAW-damaged (BAW), white mold-infected (Fungus), and white mold-infected + BAW-damaged (Fungus + BAW) peanut plants

Compound / Treatment[e]	Control	BAW	Fungus	Fungus+ BAW
Soluble protein[a]	20.3 (1.4)a	20.2 (5.0)a	23.2 (5.5)a	22.5 (2.5)a
Insoluble protein[a]	10.0 (1.0)a	8.0 (0.4)a	8.0 (0.5)a	8.4 (0.8)a
Soluble sugars[b]	99.5 (6.0)b	81.2 (3.7)a	120.3 (5.0)c	107.2 (5.1)bc
Starch[b]*	117.1 (5.0)a	111.3 (8.5)a	89.2 (4.5)b	83.6 (4.1)b
Soluble phenolics[b]	159.2 (4.7)a	154.3 (4.7)a	109.6 (9.2)b	108.4 (9.8)b
Trypsin proteinase Inhibitor[b]	12.4 (0.3)a	11.8 (0.2)a	11.8 (0.2)a	12.0 (0.4)a
Cysteine proteinase inhibitor[c]	60.1 (1.9)a	58.5 (4.6)a	63.8 (1.5)a	71.5 (1.8)b
Jasmonic acid[d]	ND	25.5 (1.4)a	16.0 (2.5)a	73.6 (10.6)b
Salicylic acid[d]	1222 (108)ab	798 (69.4)a	1024 (73.8)ab	1539 (122)b

ND= not detected

[a]Amount in mg/g fresh weight

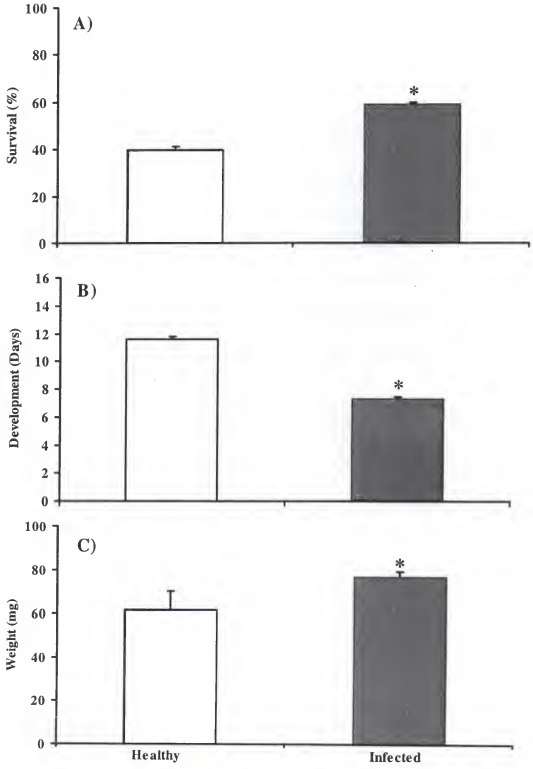
[b]Amount inμg/g of fresh weight, * Amount of glucose cleaved from starch

[c]Percent decrease in absorption relative to control reaction without plant extract

[d]Amount expressed in ng/g fresh weight

[e] Means within rows followed by the same letter are not statistically different (Tukey's mean separation test, p=0.05)

Figure 3-1. BAW larval preformance, from 3rd instar to pupae, on healthy and white mold-infected peanut in a no-choice situation: A) mean percent survival, B) mean developmental time (days) from 3rd instar to pupae, and C) mean pupal weight (mg). Light bars represent healthy control plants and dark bars represent white mold infected plants. Error bars denote 1 SE.


A)
Survival (%)
100
80
60
40
20
0
*
B)
Development (Days)
16
14
12
10
8
6
4
2
0
*
C)
Weight (mg)
100
80
60
40
20
0
*
Healthy
Infected

CHAPTER 4

EFFECT OF PEANUT PLANT FUNGAL INFECTION ON OVIPOSITION PREFERENCE BY *SPODOPTERA EXIGUA* AND ON HOST SEARCHING BEHAVIOR BY *COTESIA MARGINIVENTRIS*

Introduction

Knowledge of the factors mediating host searching behavior by insect herbivores and their natural enemies is imperative for the development and deployment of economically feasible and environmentally sound management tactics for the pests. Plant-derived volatile chemicals directly affect the behavior of herbivorous and carnivorous insects. Oviposition site selection by female herbivores is mediated for the most part by chemical cues (Renwick 1989). For example, gravid codling moths are attracted to apple tree branches with green fruit and it has been demonstrated that the orientation is in response to host emitted volatiles (Yan et al. 1999, Witzgall et al. 1999).

Herbivore-induced plant volatiles in particular have been the subject of many studies in the last 15 years because of their role in attracting natural enemies of the herbivorous species. For example, predatory mites, *Phytoseiulus persimilis,* have been found to be attracted to odor blends emanating from host plants damaged by their prey, *Tetranychus urticae* (Dicke & Sabelis 1988, Dicke et al. 1990). Also, enhanced emission of plant volatile chemicals result from *Spodoptera exigua* feeding on corn plants (Turlings et al. 1991a). These volatile emissions have been found to attract the parasitoid, *Cotesia marginiventris,* to the micro-habitat of the herbivores (Turlings et al. 1991a). In

fact, herbivore-induced volatile compounds released by plants have been shown to be the most important cues used by parasitoid wasps to locate their host caterpillars (Turlings et al.1991a, 1991b).

Volatile chemicals can also be produced by plants in response to pathogen infection. For example, infection by the fungus *Alternaria brassicae* on *Brassica rapa* seedlings induces the release of volatile compounds derived from glucosinolate degradation (Doughty et al. 1996). Also, *Phaseolus vulgaris* plants release volatile linolenic acid derivatives 15-24 h after inoculation with *Pseudomonas syringae pv. phaseolicola* (Croft et al. 1993). *Arachis hypogaea* plants infected with the white mold fungus, *Sclerotium rolfsii*, release *E*-4, 8-dimethyl-1,3,7-nonatriene, methyl salicylate and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Cardoza et al. 2002a). Previous pathogen infection on the plant has also been found to affect plant produced volatile blends in response to insect damage. The volatile blend produced when peanut plants were infected by the white mold fungus and then fed on by *S. exigua* (beet armyworm, BAW) caterpillars contained methyl salicylate and the fungus-produced 3-octanone, in addition to those emitted by healthy plants exposed to insect feeding alone (Cardoza et al. 2002a).

Many insect herbivores use plant-produced volatile compounds to locate their hosts, and their parasitoids also use plant-derived volatile chemicals to locate the herbivores. Hence, the volatiles emitted by plants in response to pathogens infection may affect oviposition site selection by herbivore females and/or the host-searching process by natural enemies. In this study I evaluated the effect of *Sclerotium rolfsii* infection on

peanut plants on the oviposition site selection by BAW and on the landing frequency and attraction of their parasitoid *C. marginiventris* to BAW damaged plants.

Materials and Methods

Plant and Insect Material

'Georgia Green' peanut seeds were provided by Drs. Tim Brenneman and Glen Raines (Coastal Plain Experiment Station, University of Georgia, Tifton, GA). Seeds were sown in pairs in 3.78-L pots (16-cm diam) containing a 1:1 (vol:vol) mixture of commercially available filter sand and Metromix 300 (Scotts-Sierra Horticultural Company, Marysville, OH). Plants were grown in an insect-free greenhouse with natural light, under Florida summer conditions (14L:10D light cycle). The greenhouse temperature was kept between 25-30°C. After emergence, seedlings were thinned to one individual per pot. Each plant received 100 ml of a 3.38 g/L liquid fertilizer solution (20-20-20 [N-P-K] Peters, W. R. Grace, Fogelsville, PA) every two weeks starting one week after emergence. At the beginning of all experiments, plants were five-weeks old and had six fully expanded leaves on the main stem and three fully developed leaves on each of two secondary branches.

Beet armyworm eggs and *C. marginiventris* cocoons were obtained from the rearing facilities at the USDA-IBPMRL, Tifton, GA. BAW were reared on a pinto-bean artificial diet following the methodology described by King and Leppla (1984). Wasps were provided with a 20% sucrose solution. All insects were kept in a biological incubator with a 14:10 L:D cycle maintained at 25 °C.

Fungal Culture

Sclerotium rolfsii (strain 80) was grown on potato dextrose agar (PDA) petri plates from original cultures provided by Dr. Tim Benneman (Coastal Plain Experiment Station, University of Georgia, Tifton, GA). Fresh cultures were started, under sterile conditions, in our laboratory by placing sclerotia harvested from the original culture in the center of PDA media plates. Culture plates were kept in a biological incubator with a 14:10 L:D cycle and maintained at 25°C and 60-70% RH° for 3 d. To inoculate the experimental plants, fungal culture plugs (5 mm diam) were cut out of the agar plate with a # 2 cork borer.

S. rolfsii Infection on Peanut

Peanut plants were infected with the fungus by distributing four culture plugs along the main stem. The plugs were positioned so the fungus was in direct contact with the stem. Fungal plugs were pressed against the stem so they remained in place. Each plant was then individually covered with a 3.78-L plastic storage bag (Ziploc DowBrands L. P., Indianapolis, IN) to provide adequate humidity and temperature conditions for fungal growth and colonization of the plant's stem. The plants were incubated for 3 d, after which time lesions of approximately 1-cm long could be observed at the point of fungal contact with the stem. After this incubation period, bags were removed from the plants 24 h before being used for the experiment. *S. rolfsii* is a non-systemic pathogen and only the stems of the plants were in contact with the fungus and therefore, the leaves that were consumed by the caterpillars were not infected by the fungus.

BAW Oviposition

To test the oviposition preference of BAW moths, three healthy and three fungus-infected plants were placed within a 4 × 2 × 3 m (L × H × W) screen cage. This cage was under natural Florida late summer environmental conditions, 25-35 °C, 80-90% RH and a light cycle of approximately 14 L:10D. Plants were distributed in two rows along the length of the cage so there was approximately a 1 m distance between plants. Healthy and infected plants were placed in an alternate fashion. Additionally, to account for any environmental differences within the cage, plants were moved so they were on opposite sides of the cages each day throughout the experiment. Sixteen 5-7 d-old adult BAW, 8 females and 8 males, were released in the center of the cage and were allowed access to the plants for 3 days. Insects were provided with cotton balls soaked in a 20% sucrose solution placed in the middle of the room as a source of food. At the end of the experiment, leaves from each treatment containing egg masses were removed from the plants and brought into the laboratory to determine the number of masses and individual eggs, with the aid of a stereo microscope, laid on plants with the two treatments. This experiment was repeated over time for a total of 6 replicates.

Response of *C. marginiventris* to Healthy and *S. rolfsii*-Infected Peanuts Damaged by BAW

To test the effect of white mold infection on peanut attraction to BAW larval parasitoids, infected and healthy peanut plants were exposed to feeding by 10 3rd instar BAW larvae for 24 h. During this time, caterpillars had free access to all leaves on the plants. After this feeding exposure, insects were removed from the plants, and one healthy and one white mold-infected plant were placed 1 m apart in a 2.5 × 0.6 × 0.6 m

(L × H × W) plexiglass cage. Ten 3-5 day-old mated female parasitoids were released in the middle of the cage. Parasitoids needed approximately 20-30 min to adjust to the new environment before responding to the plants. During this time insects were observed exiting the container in which they were introduced and interacting with each other while hanging from the top of the cage. After the adjustment period, insects were watched for an additional 15 min during which the number of landings on each plant was recorded. At the end of the observation period, insects were removed from the cage, the position of the plants was switched and a new set of insects was introduced, allowed to settle for approximately 30 min, and watched for another 15 min. This procedure was repeated twice more during the same day, with the same set of plants and with a total of four wasp sets. This experiment was repeated three times more at different times with different sets of plants and insects. All experiments were conducted in the period between 9:00 am and 4:00 pm.

To determine if the landing preference by the parasitoids was mediated by the plants' volatile emissions, I conducted dual-choice tests in a wind tunnel (Fig 4-1). The plexiglass tunnel used in this experiment was 2.5 × 0.6 × 0.6 m with an airflow of approximately 0.2 m/s (Eller et al. 1988). The tunnel was in room kept at 28 °C and approximately 80% RH. Lighting was provided by four overhead incandescent lights (90 W). Healthy and fungus-infected plants were removed from the pots and the soil around the roots was carefully removed. The roots were then wrapped with 3 plies of wet paper towels and placed inside 3.78-L plastic storage bag. The entire plant was then placed within 4-L glass jars (described below) and exposed to feeding by 15 3rd instar BAW 12 h

before the start of the experiment. Supplemental illumination for the plants was provided by a 90 W incandescent lamp from above the glass jars. Plants with each of the treatments were contained in a 4-L Mason glass jar (Alltrista Corporation, Muncie, IN) fitted with a metal lid. The jar lids were modified my drilling 2 equidistantly-distributed holes of approximately 0.6 cm diam. The holes were fitted with Teflon tubing so one would serve as an inlet and the other as an outlet for the air passing over the plants. The air outlet from each of the jars was connected to one of 2 odor sources located at the top on the upwind side of the tunnel . Odor sources were parallel to the airflow and were 30 cm apart from one another, 8 cm from the upwind end, 13 cm from each of the walls, and approximately 30 cm above the floor of the tunnel. Odors were introduced into the tunnel by a stream of humidified air passed at a rate of 500 ml/min over each plant treatment. Wasps used for this experiment were 3-5 d old mated females. To ensure the insects' response to peanut plant odors, wasps were provided a stinging experience on 2nd and 3rd instar BAW feeding on healthy peanut leaves 12 h before being used in the bioassay. Insects responding to each of the odor sources were trapped on 10-cm-diam. circles made out of light green adhesive paper (Atlantic Paste and Glue, Brooklyn, NY). A hole was cut in the center of the circle so it could be fitted around the odor source tubbing. Sets of 10 wasps were introduced approximately 1.5 m downwind and centered so that insects would come in contact with plumes from both odor sources. Insects were released 12:00-2:00 pm and were allowed to remain in the tunnel for 18-20 h, at which time the number of insects caught on each of the sources were counted and recorded. Insects within the tunnel were provided with a cotton ball soaked in a 20% sucrose solution placed at the site of release as a source of food. The odor sources were switched before each bioassay

to account for any variation in environmental conditions within the tunnel. This experiment was repeated over time, using different sets of plants and insects, to obtain a total of 8 replicates.

Statistical Analyses

Data for BAW oviposition preference and parasitoid landing and wind tunnel responses were analyzed by paired *t*-test (Proc MEANS, SAS Institute, 1996).

Results

Adult BAW oviposited significantly more on peanut plants that were infected by the white mold compared to healthy ones (Fig 4-2A, B). On average, BAW laid approximately 2-fold more eggs on peanut plants infected with the white mold fungus than they did on healthy plants. Also, the BAW larval parasitoid *C. marginiventris* landed significantly more on plants that were damaged by BAW larvae when plants were also white mold infected than when they were healthy (Fig 4-3A). On average 3 more landings were observed on plants infected with the fungus compared to healthy ones. Similarly, in the wind tunnel experiment, 3× more wasps responded to infected plant odors compared to odors originating from healthy plants (Fig 4-3B). When fungus-infected peanut plants are fed on by BAW larvae they also are more attractive to the parasitoids of the herbivores. Thus, while infected plants are favored by BAW for oviposition, and even though, BAW larval development is enhanced on such plants, white mold-infected plants do not provide protection to the herbivores against their parasitoids.

Discussion

Attraction of phytophagous insects and their natural enemies to their respective hosts involves both olfactory and visual cues. Attraction from long distances or during the scotophase , when visual cues are not available or easily perceived, may rely heavily on smell. Thus, olfactory cues are powerful and may be the most important stimuli during this phase of host searching (Huang & Renwick 1993, Renwick & Chew 1994, Turlings et al. 1990, Röse et al. 1996, Dicke & van Loon 2000). After the insect has alighted on the plant, the evaluation of physical and chemical characteristics of the plant tissue will help in the final decision making by the phytophagous insects before either feeding or oviposition takes place. Plant cells contain large numbers of different chemicals and some may stimulate while others may deter insects from feeding or ovipositing on a given plant.

Peanut plants infected with the white mold fungus, *S. rolfsii*, have higher levels of soluble sugars and lower levels of soluble phenolics, which may result in increased feeding preference and performance by BAW (Cardoza et al. 2002a, b). These internal biochemical changes may also explain the increased BAW oviposition on fungus-infected peanut observed in the present study. In addition to changes in internal physiology caused by pathogen attack, plants may also release volatile substances. For example, peanut plants infected with the white mold fungus, release a set of compounds that differs significantly from that of healthy plants and plants that are damaged by BAW (Cardoza et al. 2002a). Ovipositing choice by herbivores can be affected by the plant's chemical composition (Deithier 1988, Ahman, 1985). For example, *S. littoralis* preferred to

oviposit on cotton plants that had been fed on by conspecific (Anderson & Alborn 1999). On the other hand, when given a choice between undamaged and conspecific-damaged cotton plants, *Trichoplusia ni* females were attracted to the odors of the damaged, but preferred to oviposit on the healthy plants (Landolt 1993). Thus, the internal and external expressions of biochemical changes induced in plants by pathogen infection may play a role in oviposition site selection by herbivore females. In the present study I found that BAW laid more eggs on white mold-infected peanut plants than healthy ones. In this experiment, however, olfactory, visual and tactile cues could all have contributed to the insect's final selection. Therefore, additional experiments need to be performed to determine if oviposition site selection by these insects was due to the difference in volatile emissions between healthy and white mold infected peanut plants.

Volatile plant chemical compounds play a major role in guiding the host searching behavior by natural enemies (Turlings et al. 1991, Tumlinson et al. 1993, Röse et al. 1998, Kessler & Baldwin 2001). These chemical signals may derive from the herbivore host (Vinson 1976, van Alphen & Vet 1986), from the intact plants (Vinson 1976), or from the herbivore-damaged plant. Herbivores have evolved ways to evade their natural enemies, thus it can be expected that they would emit very little in terms of odors that parasitoids could use to locate them. Thus, long distance location by parasitoids seems to be dependent upon information provided by other sources such as the host plants upon which the herbivores are feeding. Female herbivores select oviposition sites that not only provide the best suitable food for their brood, but also minimize exposure to natural enemies (Faeth 1986, Godfray 1994). Since BAW not only oviposited more on, but

larvae fed more and develop better on fungus- infected peanuts (Cardoza et al. 2002b), could it also be that feeding on infected plants helps BAW avoid natural enemies? To test this hypothesis, I conducted dual choice experiments to determine if peanut plant infection by *S. rolfsii* had any negative effect on the parasitoid's ability to find diseased plants fed on by BAW. *Cotesia marginivetris* were observed landing with significantly higher frequency on peanut plants that were infected by the fungus and then exposed to BAW feeding than on plants exposed to BAW alone. White mold-infected plants release greater amounts of some volatiles compounds compared to healthy ones (Cardoza et al. 2002a). We believe that the greater release of volatile compounds associated with BAW leaf consumption may be responsible for the preferential landing on, and greater response to white mold-infected peanut plants by *C. marginiventris*.

Manipulation of insect herbivores and their natural enemies has potential applications for the control of agricultural pests in today's agriculture. To exploit this potential in an economical and practical manner we must first understand the biology and behavior of the insects, and their interactions with their respective hosts. Several studies have evaluated the effect of plant pathogen infection on feeding and performance of herbivorous insects (Kluth et al. 2001, Hatcher et al. 1995, Moran 1998). A few of them have also evaluated the effect of plant disease on oviposition preference by the herbivore (Friedli & Bacher 2001). However, to our knowledge, this is the first time that the effect of pathogen-induced biochemical changes in plants on parasitoid behavior has been evaluated.

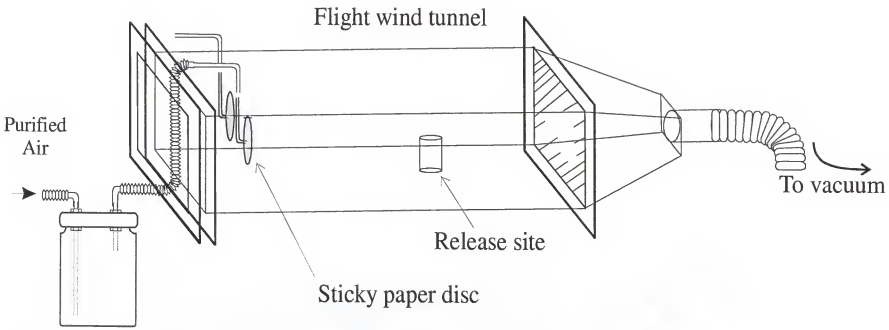


Figure 4-1. Wind tunnel system used to test the effect of volatile emissions from healthy and fungus-infected peanut plants on the response of the parasitoid *Cotesia marginiventris* to BAW-damaged plants.

Figure 4-2. Oviposition preference by *Spodoptera exigua* choice tests with healthy (white bars) and white mold-infected (dark bars) peanut plants. A) Average number of egg masses per plant, and B) Average number of eggs per plant. Error bars denote 1 SE and * denotes significant differences (paired *t*-test, $P \leq 0.05$).

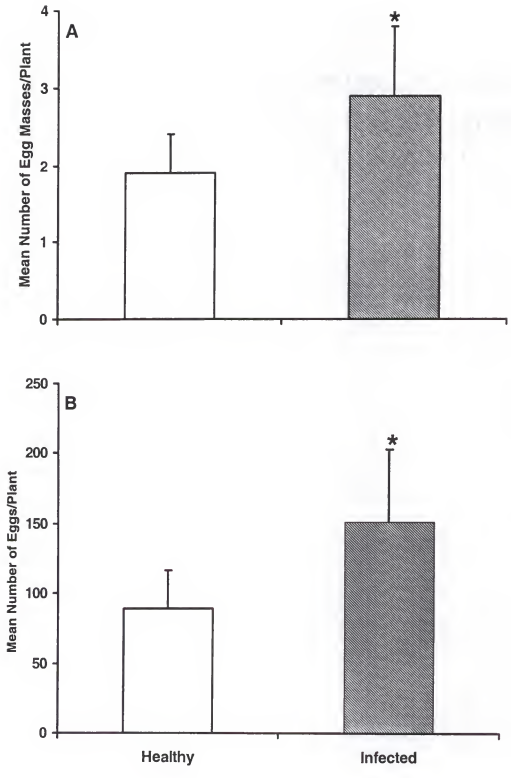
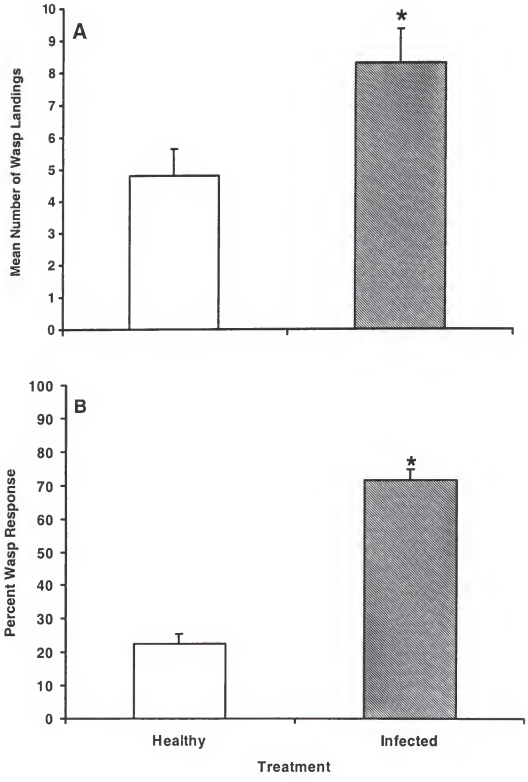

A
Mean Number of Egg Masses/Plant
0
1
2
3
4
*
B
Mean Number of Eggs/Plant
0
50
100
150
200
250
*
Healthy
Infected

Figure 4-3. Response of the BAW parasitoid *Cotesia marginiventris* choice tests with healthy (white bars) and white mold-infected (dark bars) plants fed on by BAW. A) Landing preference on plants, and B) Wind tunnel response to plant odors. Error bars denote 1 SE and * denotes significant differences (paired *t*-test, $P \leq 0.05$).


A
Mean Number of Wasp Landings
10
9
8
7
6
5
4
3
2
1
0
*
B
Percent Wasp Response
100
90
80
70
60
50
40
30
20
10
0
*
Healthy
Infected
Treatment

CHAPTER 5

INDUCTION OF VOLATILE COMPOUNDS AND SIGNALING HORMONES BY BACTERIAL INFECTION AND INSECT HERBIVORE DAMAGE ON PEPPER PLANTS

Introduction

Plants possess a number of chemical defense mechanisms that are often triggered by herbivore and/or pathogen attack. These chemical defenses can directly modify the development and survival of the attacking organism (Mür et al. 1997), or can serve as attractants to natural enemies of the pest (i.e., release of herbivore-induced synomones) (Turlings & Tumlinson 1991, Turlings et al. 1991a, 1993). In addition to the production of internal defense compounds in response to pest attack, plants may also produce volatile substances that are released externally. Fort example peanut plants release a unique profile of such compounds in response to attack by either herbivores or pathogens (Cardoza et al. 2002a).

Examples of pathogens eliciting the production and release of volatiles from the affected plant hosts include the emission of volatile products from glucosinolate degradation by *Brassica rapa* seedlings when infected by the fungus *Alternaria brassicae* (Doughty et al. 1996). In another study with beans (*Phaseolus vulgaris* L.), release of volatile linolenic acid derivatives ensued after inoculation with *Pseudomonas syringae* pv. *phaseolicola* (Croft et al. 1993). Lipoxygenase-derived volatiles are also emitted from discs of pepper, *Capsicum annuum* L., leaves after infiltration with a virulent strain of the

bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria* (XCV) (Buonaurio & Servili 1999).

The regulation of the defense mechanisms that help plants cope with multiple stress factors remains to be elucidated. Similarly, it is not yet clear what defense pathways are involved in the production of volatile chemicals by plants in response to insect and pathogen attack. Recent studies suggest that activation of plant defenses by pathogens interferes with plant defenses against herbivorous arthropods and vice-versa (Karban et al. 1987, Fidantsef et al. 1999, Stout et al. 1999, Bostock 1999, Felton et al. 1999). In light of these possible conflicts between the pathogen- and herbivore-induced plant defenses, it has become apparent that studies on the interactions between insect and pathogen species, and the induced chemical changes that such interactions have on the host plant are lacking.

The goal of this study was to investigate the possible production of volatile compounds by pepper plants at different times during infection by *Xanthomonas campestris* pv. *vesicatoria* (XCV), and to evaluate the effect of simultaneous bacterial/herbivore challenge on the volatile emission by these plants. The level of the plant hormones jasmonic acid (JA) and salicylic acid (SA), which are believed to be involved in herbivore-and pathogen-defense signaling in plants, was measured in plants treated with XCV pepper race 3 (XCVP3) and beet armyworm, *Spodoptera exigua*, BAW individually and in combination.

Materials and Methods

Plant and Insect Material

'Early CalWonder' pepper seeds (Grimes, Concord , OH) were sown in pairs in 1-gal pots (16-cm diam) containing Metromix 300 (Scotts-Sierra Horticultural Company, Marysville, OH). Plants were grown in an insect-free greenhouse with natural light, under Florida summer conditions (14L:10D light cycle). The greenhouse temperature was maintained between 25-30 °C. After emergence, seedlings were thinned to one individual per pot. Each plant received 100 mL liquid fertilizer (20-20-20 [N-P-K] Peters, W. R. Grace, Fogelsville, PA) every two weeks starting on the first week after emergence. Six-week old pepper plants with 8 fully developed leaves were used in all experiments. Eggs of beet armyworm, *S. exigua*, eggs were obtained from the rearing facilities at the USDA-IBPMRL, Tifton, GA. Larvae were reared on a pinto bean-based artificial diet following the methodology described by King and Leppla (1984). Insects were kept in a biological incubator with a 14:10 L:D cycle and maintained at 28 °C. Third instar larvae were used in all experiments.

Pathogen Culture and Plant Inoculation

The initial culture of *Xanthomonas campestris* pv. *vesicatoria* (XCVP3, pepper race 3, strain 88-5), was obtained from Jeffrey B. Jones (Department of Plant Pathology, University of Florida, Gainesville, FL) and was then grown in nutrient agar (NA) petri plates. Subsequent cultures were started in our laboratory using nutrient broth and were stored in 15% glycerol at -70 °C for later use. Viable cells for plant inoculation were obtained by incubating 100 µl of the frozen cultures in 50-ml conical centrifuge tubes

containing 15 ml nutrient broth. Tubes were placed in a 200 rpm orbital shaker within a biological incubator kept at 28 °C for 18 h. Cells were harvested by spinning at 3,000 × g for 10 min and pellets were re-suspended in tap water. Bacterial cell concentration was estimated by measuring absorbance with a spectrophotometer set at 600 nm. Concentration was then adjusted to 10^7 colony forming units/ml with water and 400 µL/L of Silwett L-77 was added to help cell penetration of the leaves. Plants were inoculated by dipping their aerial portions in the bacterial suspension for 20 s. Control plants were mock inoculated by dipping in a mixture of water and Silwett L-77 without bacterial cells.

Collection of Volatile Compounds from *Xanthomonas* and BAW-Damaged Peppers

Plant treatments consisted of a) control (uninfected/undamaged), b) BAW-damaged, c) XCV-infected and d) XCV-infected plus BAW damage. Bacterial inoculation of plants was the same as described in the previous section. BAW-damaged plants were exposed to feeding by six 3rd instar larvae within the volatile collection chambers for 12-h before the start of the first sampling period. Thus, in this experiment, plants exposed to bacterial infection had been inoculated 12 h before the start of the first collection period.

The aerial portions of the plants were contained within the glass sleeves of guillotine type volatile collection chambers (Analytical Research Systems Inc., Micanopy, FL) which rested on Teflon bases with an opening in the center that surrounded the plant stems (Röse et al. 1996). Purified air was pushed in at the top of the volatile collection chamber at a rate of 5 L min^{-1}. Air within each of the chambers was sampled daily, at a

rate of 1 L min^{-1}, for 4 d in three consecutive periods 1) 6:00 am-12:00 pm, 2) 12:00 pm-6:00 pm, and 3) 6:00 pm-6:00 am. Compounds emitted were collected at the downwind end of the chambers in 25 mg Super Q (800-100 mesh) adsorbent traps (Alltech, Deerfield, IL). The experiment was set up in single replicates and repeated on different days for a total of 6 replicates.

To evaluate the effect of disease development on the plant's ability to produce volatiles in response to insect damage, a second experiment was conducted in which the plants were inoculated with XCVP3 4 d before being used in the experiment. This yielded data for days 4-8 after XCVP3 infection. Plants damaged by BAW were exposed to the insect's feeding 12 h before the first volatile collection period. The volatile collection procedure was the same as outlined above. This experiment was also repeated on different days for a total of 6 replicates.

Extraction and Analysis of Volatile Samples

Compounds from individual adsorbent traps were eluted with 170 µL dichloromethane (GC/GC-MS Solvent, B&J, AlliedSignal, Inc, MI), and then 400 ng each of *n*-octane and nonyl acetate were added to each eluted sample as internal standards. The samples were analyzed by gas chromatography with flame ionization detection (HP5890 Gas Chromatograph, HP7673 auto sampler, Hewlett Packard, Palo Alto, CA) equipped with a 15-m × 0.25 mm ID, 0.25-µm film thickness DB-1 capillary column (Quadrex, New Haven, CT). The splitless mode injector system was set at 220°C, the column oven was held at 40°C for 1 min after injection and then programmed

at 14°C min^{-1} to 180°C. The carrier gas used was helium at an average flow velocity of 19 cm s^{-1}.

For identification of compounds, selected samples were analyzed via GC/MS (HP 6890 Gas Chromatograph equipped with 30 m × 0.25 mm ID, 0.25-µm film thickness HP-5 capillary column, interfaced to a 5973 Mass Selective Detector, Hewlett Packard, Palo Alto, CA) in both electron impact and chemical ionization modes. The column was held at 40°C for 1 min after injection and then programmed at 10°C min^{-1} to 180°C. The carrier gas used was helium at an average flow velocity of 30 cm s^{-1}. Isobutane gas was used as the reagent gas for chemical ionization, and the ion source temperature was set at 250°C. Individual compounds were identified by comparing their retention times to that of commercially obtained authentic samples and by comparing their mass spectra against those available in a database from the Environmental Protection Agency/ National Institute of Standards and Technology.

Levels of JA and SA in Plants

Plants that were at the tenth true-leaf stage were either left non-inoculated/undamaged to serve as controls or exposed to either XCVP3-infection alone, BAW-damage alone, or a combination of XCVP3 + BAW-damage. For plants receiving BAW damage, two 3rd instar larvae were caged on small petri dish clip cages on to the 3rd, 6th, and 9th leaves of the plant immediately following inoculation with XCVP3. A disc of approximately 1.5 cm in diameter was cut daily for 4 consecutive days with a # 6 cork borer from each of these leaves starting 24 h after inoculation with XCVP3. Leaf tissue was weighed and immediately frozen in liquid N_2 and kept in a -70 °C freezer until

needed for analysis of hormone levels. Leaves sampled in all treatments were the equivalent to those exposed to insect feeding in the BAW damaged plants.

Jasmonic acid and free and conjugated SA extraction and analysis were performed following a method by Engelberth et al. 2002. Briefly, frozen tissue was ground in liquid N_2 with a glass pestle to obtain a fine powder. The samples were then extracted with an acetone/citric acid mixture and deuterated SA and dihydroJA (Sigma Chemical Co., St Louis, MO) were added as internal standards. The mixture was vortexed for 30 s and then sonicated for 20 min. The samples were then centrifuged at 3000 × g for 5 min. The supernatants were transferred to new tubes and the acetone in each of the samples was evaporated under a stream of air. Samples were then extracted twice with 100% diethylether, transferring the upper phase to a new tube after each extraction. The collected upper phases were dried under an stream of air until all water had evaporated and methylated. The methanolysis procedure was accomplished by adding 30 µL of a 2:1 MeOH: HCl mixture to each of the samples, vortexing for 30 s and then incubating at 70 °C for 45 min. For extraction of conjugated SA, each of the leftover lower phases were acidified by adding 10 µL of concentrated HCl, vortexed for 30 s and incubated at 99 °C for 1 h. Samples were allowed to come to room temperature, and then extracted with diethylether, dried down under an air stream, and methylated as above. After methanolysis, each conjugated SA sample received 90 µL of a 50 mM citric acid (tri-sodium salt) in water to neutralize the remaining HCl. The methylated products from all extractions were collected using the same adsorbent traps used in the volatile collection experiment. The air from each of the sample vials was forced through the adsorbent

matrix by applying vacuum at the far most end of the trap. To speed up the volatilization process, sample vials were placed on a 40 °C heating block. Volatiles were collected until all the liquid had evaporated from the bottom of the vials. Samples were analyzed by gas chromatography-mass spectrometry (GC-MS) on a Hewlett-Packard (HP) 6890 GC (He carrier gas; 0.7 ml min^{-1}; splitless injector 240 °C, injection volume 2 µl) with a HP-5MS column (5% phenyl methyl siloxane , 30m x 250mm i.d. x 0.25 mm film thickness) with the temperature programmed from 40 °C (1 min hold) at 10 °C min^{-1} to 240 °C (hold for 15 min). The GC was coupled to a HP 5973 quadrapole-type mass selective detector with transfer line, source, and quadrapole temperatures of 230 °C, 230 °C and 150 °C, respectively. Chemical ionization with isobutane as the reaction gas generated predominantly M+1 parent ions scanned at a range of 60-500 amu.

Statistical Analyses

Data for total volatile emission and JA and SA levels were analyzed using ANOVA (Proc GLM, SAS Institute 1996). Significant ANOVAs were followed by Tukey's mean separation test.

Results

In general, volatile emission in all plants showed a diurnal pattern with peak release during period 1 (6:00 am-12:00 pm) or period 2 (12:00-6:00 pm). In the first experiment, volatile release 1-4 days after bacterial inoculation was measured. Healthy pepper plants released α- and β-pinene, α-humulene, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and relatively small amounts of a number of other compounds (Fig 5-1a). BAW-damaged plants released a wide array of compounds, including monoterpenes, a

large number of lipoxygenase products, indole, the homoterpenes *(E)*-4, 8-dimethyl-1,3,7-nonatriene and *(E,E)*-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and number of sesquiterpenes (Fig 5-1c). The volatile profile of pepper plants damaged by BAW alone was quantitatively and qualitatively different from that of healthy and XCVP3 plants (Fig 5-1a, 5-1b, 5-1c). Plants infected with XCVP3 alone emitted *(E)*-2 hexenal, methyl salicylate, and *(E,E)*-4,8,12-trimethyl-1,3,7,11-tridecatetraene in quantities that were significantly larger than those present in healthy plants (Fig 5-1b). XCVP3-infected peppers exposed to BAW-feeding released all the compounds present in profiles of peppers damaged by BAW alone, in addition to methyl salicylate (Fig 5-1d). Methyl salicylate was only detected in emissions from plants that were infected with XCVP3 and not in that of plants damaged by BAW alone. Plants infected with the bacteria released volatiles in quantities comparable to healthy/undamaged plants (Figs 5-1b, 5-1d).

In the second experiment volatiles were collected and analyzed 4-7 d after bacterial inoculation. Healthy pepper plants released α-pinene, β-pinene, caryophyllene and α-farnesene (Fig 5-2a). BAW-damaged plants released a wide array of compounds and this profile was quantitatively and qualitatively different from that of healthy, XCVP3- and XCVP3+BAW-damaged plants (Fig 5-2c). Volatile emissions from plants infected with XCVP3 alone consisted mainly of methyl salicylate (Fig 5-2b). Additionally, XCVP3 plants released amounts of β-caryophyllene, α-farnesene, and β-selinene that were significantly larger than those present in healthy control plants, but similar to those emitted by plants damaged by BAW alone (Fig 5-2b). XCVP3-infected peppers exposed to BAW-feeding emitted all the compounds present in profiles of

peppers damaged by BAW alone, but in lower quantities, with the exception of α-farnescene which was one of the most prominent compounds along with methyl salicylate (Fig 5-2d). Methyl salicylate was only present in emissions from plants that were infected with XCVP3 (Fig 5-3a, b).

Total volatile emission by plants infected by the bacteria alone was similar to that of control plants in the first experiment (Fig 5-4a), but increased significantly, to levels that were comparable to those emitted by XCVP3/BAW plants, during the second experiment (Fig 5-4b). Total volatile emission by XCVP3-infected plants exposed to BAW was comparable to that of plants damaged by BAW alone in the first experiment (Fig 5-4a). However, 4 days after inoculation (second experiment), XCVP3-infected plants released less volatiles than healthy plants in response to BAW damage (Fig 5-4b). Thus, it appears that the ability of plants to produce volatiles in response to herbivore damage is negatively affected as the XCVP3 infection progresses in the plant. It was noted, that the decrease in total volatile production by plants under simultaneous bacterial and BAW-attack was preceded by a sharp increase in the release of methyl salicylate by these plants (Fig 5-3).

Levels of JA and free SA did not differed significantly across treatments throughout the duration of the sampling period (Fig 5-5a, 5-5b). On the other hand, conjugated SA levels in XCVP3 and XCVP3/BAW plants were significantly higher than that of control and BAW-damaged plants on days 2, 3 and 4 (Fig 5-5c). Although, on day 3, the level of conjugated SA increased dramatically in plants treated with the

XCVP3/BAW complex (Fig 5-5c), levels of conjugated SA in control and BAW-damaged plants did not differ significantly throughout the experiment (Fig 5-5c).

Discussion

In recent years, studies that have focused on the interactions that occur between insect herbivores, plant pathogens and their hosts have provided an insight into the complexity of the mechanisms used by plants in their defense against individual insect and pathogen attack. Secondary compounds produced by plants in response to insect and pathogen attack include volatile substances. Insect-induced volatile compounds attract natural enemies of the insects and may directly affect the performance of the organism responsible for the attack . Similarly, pathogen-induced volatile substances may directly hinder pathogen development (Zeringue & mcCormick 1989, 1990, Zeringue et al. 1996, Cardoza et al. 2002a). Different stress factors may induce the release of different volatile profiles. For example, volatiles emitted in response to pathogen infection are qualitatively and quantitatively different from those emitted in response to insect herbivore damage on the same plant (Cardoza et al. 2002a). Volatile profiles are also affected by the combination of factors afflicting the plant at any given moment (Cardoza et al. 2002a).

In the present study I found that pepper plant volatile profiles can be differentially induced by bacterial infection and insect herbivore damage alone or in combination. I also found that plants in the early stages of bacterial infection produced greater quantities of volatiles than healthy plants when both were exposed to BAW feeding. However, 4 days after bacterial inoculation, the total volatile release by diseased plants fell below that of

healthy plants exposed to BAW feeding. Interestingly, this decrease in BAW-induced volatile release coincided with an increase in methyl salicylate emission from XCVP3-infected plants. Furthermore, analysis of defense signaling hormones revealed that this decrease in insect-induced volatile emission was preceded by a significant increase in conjugated SA levels in plants under simultaneous XCVP3/BAW attack. Curiously, this increase in SA levels did not result in a significant reduction of the JA levels in plants simultaneously exposed XCVP3 and BAW. In contrast, recent studies have suggested that induction of the SA pathway by pathogens interferes with JA-mediated defenses against herbivores (Karban et al. 1987, Fidantsef et al. 1999, Stout et al. 1999, Bostock 1999, Felton et al. 1999).

It is now well established that both, the lipoxygenase pathway leading to jasmonate production, and the salicylic acid pathway are involved in direct plant defense against pathogen invasion (Wasternack & Parthier 1997, Thomma et al. 1998). However, recent studies have also suggested that the JA and SA biochemical pathways may interfere with one another (Karban et al. 1987, Fidantsef et al. 1999, Stout et al. 1999, Bostock 1999, Felton et al. 1999). In fact, acetylsalicylate and SA have been reported to inhibit lipoxygenase-induced plant defenses by preventing JA biosynthesis (Peña-Cortes et al. 1993, Doares et al. 1995). Contrastingly, in my study, SA and JA were both detected in pathogen and BAW-damaged pepper plants. We did not observe a significant decrease in JA levels in XCVP3/BAW plants, even at the maximum level of SA accumulation on day 3. Similarly, peanut plants under simultaneous fungal and insect attack had higher levels of both JA and SA compared to plants under attack by either organism alone

(Cardoza et al. 2002a). The mechanisms involved in plant volatile synthesis and their regulation remain to be clearly established. Products from the lipoxygenase pathway have been shown to elicit volatile production in plants (Koch et al. 1999, Dicke et al. 1999, Rodriguez-Saona et al. 2001). Thus, LOX may be one of the biochemical pathways involved in volatile production. Based on the findings that allamethicin, a fungal-derived elicitor, induced volatile emissions and increased JA and SA levels in lima beans, Engelberth et al. (2001) have proposed that both biochemical pathways interact with each other to regulate volatile emissions in these plants.

In summary, the data presented herein support the idea of an orchestrated interaction between the levels of JA and SA to regulate the induction and emission of volatile organic compounds by pepper plants under bacterial- and insect herbivore-attack. This is the first time in which the levels of both JA and SA have been correlated with plant emission of volatile compounds in response to individual and synchronous pathogen- and insect herbivore- attack. Studies such as these are necessary to help unravel the complex interactions between defense pathways that help plants cope with multiple stress factors. Further experiments are necessary to determine the JA/SA ratios necessary for volatile induction and/or inhibition in this plant-herbivore-pathogen system as well as others. Additionally, the hierarchy governing plant defense responses when under attack by pathogenic and herbivorous organisms merits further attention.

Figure 5-1. Mean volatile emissions from pepper plants representative of the early stages of bacterial infection (days 1-4): A) Uninfected/undamaged control, B) XCVP3-infected, C) BAW-damaged, and D) XCVP3-Infected + BAW-damaged plants. Day 2, period 2 (12:00 pm-6:00 pm). Error bars denote 1 SE.

List of Compounds: 1) *(E)*-2-hexenal; 2) *(Z)*-3-hexen-1-ol; 3) α-pinene; 4) β-pinene; 5) *(Z)*-3-hexenyl acetate; 6) β-myrcene; 7) *(E)*-2-hexenyl acetate; 8) limonene; 9) eucalyptol; 10) β-ocimene; 11) *(Z)*-3-hexenyl propionate; 12) linalool ;13) *(E)*-4, 8-dimethyl-1,3,7-nonatriene; 14) *(Z)*-3-hexenyl isobutyrate; 15) methyl salicylate; 16) *(E)*-2-hexenyl butyrate; 17) *(Z)*-3-hexenyl butyrate; 18) 2-methyl-hexyl butyrate; 19) 2-phenyl-ethyl formate; 20) indole; 21) *(Z)*-3-hexenyl valerate; 22) *(E)*-2-hexenyl valerate; 23) *(Z)*-3-hexenyl tiglate; 24) *(Z)*-jasmone; 25) *(Z)*-3-hexenyl caproate; 26) *(Z)*-3-hexenyl hexenoate; 27) β-caryophyllene; 28) α-humulene; 29) β-farnesene; 30) β-selinene**; 31) δ-cadinene **; 32) β-elemene **; 33) α-selinene **; 34) α-farnesene; 35) nerolidol; 36) *(E,E)*-4,8,12-trimethyl-1,3,7,11-tridecatetraene; 37) *(Z)*-3-hexenyl-phenyl acetate

**No synthetic standards available, identification is based on National Institute of Standards and Technology library spectral match only.

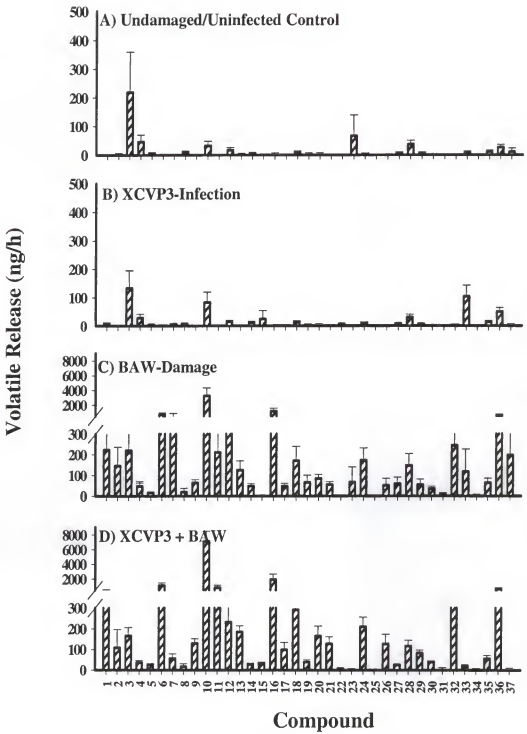

A) Undamaged/Uninfected Control
B) XCVP3-Infection
C) BAW-Damage
D) XCVP3 + BAW
Volatile Release (ng/h)
Compound

Figure 5-2. Mean volatile emissions from pepper plants representative of advanced stages of bacterial infection (days 4-7): A) Uninfected/undamaged control, B) XCVP3-infected, C) BAW-damaged, and D) XCVP3-Infected + BAW-damaged plants. Day 6, period 2 (12:00 pm-6:00 pm). Error bars denote 1 SE.

List of Compounds: 1) *(E)*-2-hexenal; 2) *(Z)*-3-hexen-1-ol; 3) α-pinene; 4) β-pinene; 5) *(Z)*-3-hexenyl acetate; 6) β-myrcene; 7) *(E)*-2-hexenyl acetate; 8) limonene; 9) eucalyptol; 10) β-ocimene; 11) *(Z)*-3-hexenyl propionate; 12) linalool ;13) *(E)*-4, 8-dimethyl-1,3,7-nonatriene; 14) *(Z)*-3-henenyl ibutyrate; 15) methyl salicylate; 16) *(E)*-2-hexenyl butyrate; 17) *(Z)*-3-hexenyl butyrate; 18) 2-methyl-hexyl butyrate; 19) 2-phenyl-ethyl formate; 20) indole; 21) *(Z)*-3-hexenyl valerate; 22) *(E)*-2-hexenyl valerate; 23) *(Z)*-3-hexenyl tiglate; 24) *(Z)*-jasmone; 25) *(Z)*-3-hexenyl caproate; 26) *(Z)*-3-hexenyl hexenoate; 27) β-caryophyllene; 28) α-humulene; 29) β-farnesene; 30) β-selinene**; 31) δ-cadinene **; 32) β-elemene **; 33) α-selinene **; 34) α-farnesene; 35) nerolidol; 36) *(E,E)*-4,8,12-trimethyl-1,3,7,11-tridecatetraene; 37) *(Z)*-3-hexenyl-phenyl acetate

**No synthetic standards available, identification is based on National Institute of Standards and Technology library spectral match only.

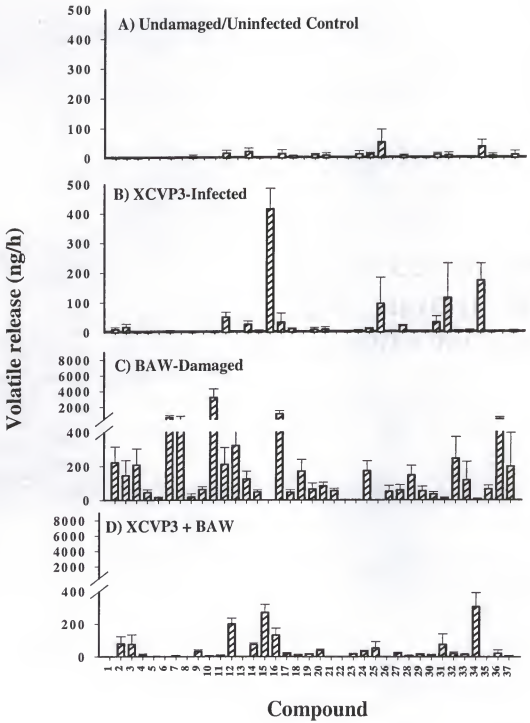

A) Undamaged/Uninfected Control
B) XCVP3-Infected
C) BAW-Damaged
D) XCVP3 + BAW
Volatile release (ng/h)
Compound

Figure 5-3. Mean methyl salicylate emission by pepper plants infected with XCVP3 alone or in combination with BAW damage. A) Experiment 1 (days 1-4 d after inoculation) and B) Experiment 2 (days 4-7 after inoculation). Error bars indicate 1 SE.

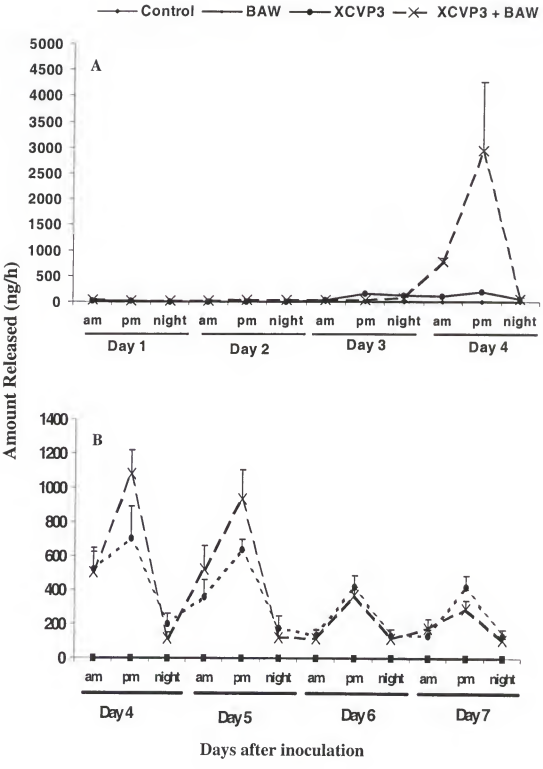

Control
BAW
XCVP3
XCVP3 + BAW
A
B
Amount Released (ng/h)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1400
1200
1000
800
600
400
200
0
am pm night am pm night am pm night am pm night
Day 1
Day 2
Day 3
Day 4
Day 4
Day 5
Day 6
Day 7
Days after inoculation

Figure 5-4. Mean total volatile emission by pepper plants A) Days 1-4 after bacterial inoculation, B) Days 4-7 after bacterial inoculation. Error bars denote 1 SE.

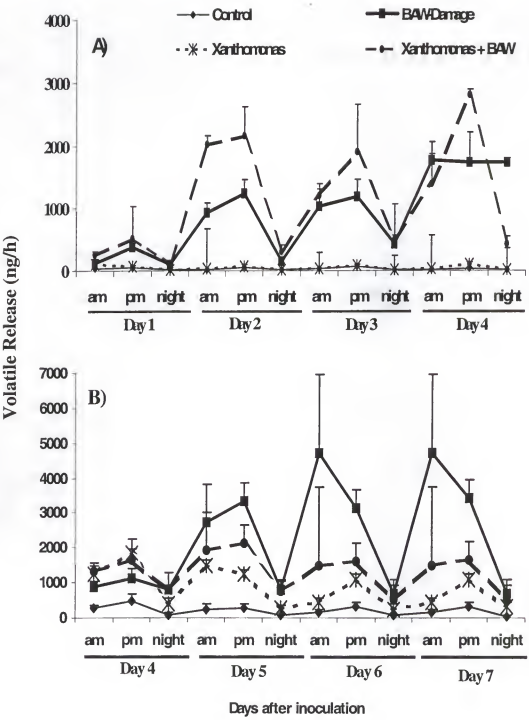
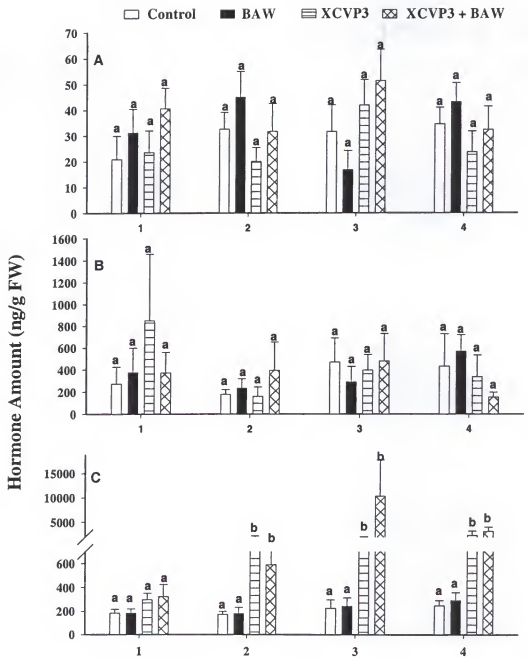

Control
BAW-Damage
A)
Xanthomonas
Xanthomonas+BAW
4000
3000
2000
1000
0
am pm night am pm night am pm night am pm night
Day 1
Day 2
Day 3
Day 4
Volatile Release (ng/h)
B)
7000
6000
5000
4000
3000
2000
1000
0
am pm night am pm night am pm night am pm night
Day 4
Day 5
Day 6
Day 7
Days after inoculation

Figure 5-5. Mean levels of signaling hormones in pepper plants under individual and simultaneous attack by XCVP3 and BAW: A) Jasmonic Acid, B) Free salicylic acid, and C) Conjugated salicylic acid. Error bars denote 1 SE. Bars within days headed by the same letter are not significantly different (Tukey's mean separation test, P=0.05)


Control
BAW
XCVP3
XCVP3 + BAW
Hormone Amount (ng/g FW)
A
B
C
1
2
3
4

CHAPTER 6
SUMMARY AND CONCLUSIONS

Release of volatile compounds by plants in response to attack by herbivores, and the role of such compounds in attracting parasitoids of the herbivores, have been studied extensively in recent years. In contrast, the emission of volatiles in response to pathogen invasion has been the subject of a limited number of studies. No studies have been conducted to examine the induction of plant volatile emission by either herbivore damage and pathogen infection on the same plant species using whole plants. Thus, there is no clear knowledge of whether the regulation of volatile production in response to these organisms is affected in any way by the simultaneous attack of herbivores and phytopathogens on the same plant. Additionally, the effect that pathogen infection might have on the plant's ability to attract natural enemies of herbivorous insects has not been studied previously.

In chapter 2, I found that peanut plants, *Arachis hypogaea*, infected with white mold, *Sclerotium rolfsii*, emitted a blend of organic compounds that differed both quantitatively and qualitatively from the blend emitted from plants damaged by beet armyworm (BAW), *Spodoptera exigua*, larvae or from uninfected, undamaged plants. Attack by BAW induced release of lipoxygenase products (hexenols, hexenals and hexenyl esters), terpenoids and indole. The plant-derived compound methyl salicylate and the fungal-derived compound 3-octanone were found only in head space samples

from white mold-infected plants. White mold-infected plants exposed to BAW damage released all the volatiles emitted by healthy plants fed on by BAW in addition to those emitted in response to white mold infection alone. When BAW larvae were given a choice of feeding on leaves from healthy or white mold infected plants, they consumed larger quantities of the leaves from infected plants (Chapter 2). Exposure to commercially available (*Z*)-3 hexenyl acetate, linalool and methyl salicylate, compounds emitted by white mold infected plants, significantly reduced the growth of the white mold in solid-media cultures. Thus emission of these compounds by infected plants may constitute a direct defense against this pathogen. To my knowledge, this is the first *in planta* study in which the production of volatiles by a single host plant in response to both insect herbivores and pathogens has been evaluated simultaneously. This is also the first time that the effect of previous pathogen infection on the production of plant volatiles in response to insect damage has been studied. The study of plant volatile defenses may improve our understanding of plant resistance mechanisms to disease and insect herbivores. The identification of specific pathogen- and herbivore-induced plant volatiles will greatly contribute to the development, improvement and implementation of host-plant resistance and other control methods for insect and pathogen pests.

In chapter 3, I wanted to further investigate the effect of fungus-induced biochemical changes in peanut on the biology of my herbivorous insect species (BAW). In the studies discussed in this chapter, I determined that 3rd instar BAW caterpillars allowed to feed on *S. rolfsii*-infected plants had significantly higher survival, produced significantly heavier pupae, and had shorter time to pupation than those allowed to feed

on healthy plants. Leaf tissue from white mold-infected peanut plants contained similar levels of soluble and insoluble protein, but significantly higher levels of soluble sugars. In addition, white mold-infected plants had significantly lower starch content and total soluble phenolics compared to leaves from healthy plants. Levels of jasmonic acid were similar in plants attacked by either the fungus or BAW, but were significantly higher in plants that were infected by the fungus and then fed on by BAW. Salicylic acid (SA) levels in fungus-infected plants were not significantly different from those of control plants. However, levels of SA in plants damaged by BAW alone were significantly lower than those of plants under simultaneous attack by the fungus and BAW. Most studies conducted on the induction of plant defenses have focused on the effect of individual pest species; however, under natural field conditions, plants may often be under attack by different insect and pathogen pest species, or combinations thereof, at any given time. Understanding the innate mechanisms of defense in plants against multiple pests is vital for future development of resistance in crops. The data presented here contribute towards a better understanding of the effects of the interactions between insect and pathogen pests on their host plants. This type of information is important for the development of tools to prevent, manage, and control pests in agricultural environments.

The biochemical changes induced in plants by pathogen infection may also affect the behavior of adult insects in the second (herbivorous) and third (carnivorous) trophic levels. Thus, in chapter 4 I tested the effect of peanut stem infection by the white mold fungus on the oviposition preference of BAW and on the host searching behavior by the BAW larval parasitoid *Cotesia marginiventris*. I found that in choice tests adult BAW

oviposited more on white mold infected plants than on healthy plants. When plants were exposed to BAW feeding, the parasitoid *C. marginiventris* landed more frequently on infected than on healthy plants. I conducted wind tunnel choice experiments to determine if the more frequent landing by the wasps was mediated by the volatiles emitted by healthy and white mold infected plants in response to BAW damage. In these wind tunnel experiments wasps were more responsive to volatiles from plants infected with the white mold compared to healthy ones, when plants were exposed to damage by BAW caterpillars. Thus, white mold-infected peanut plants are more suitable for BAW oviposition, but, when damaged by BAW, plants are also more attractive to one of the BAW natural enemies. Manipulation of insect herbivores and their natural enemies has potential applications for the control of agricultural pests in today's agriculture. To exploit this potential in an economical and practical manner I must first understand the biology and behavior of the insects, and their interactions with their respective hosts. Several studies have evaluated the effect of plant pathogen infection on feeding and performance of herbivore insects. A few of them have also evaluated the effect of plant disease on oviposition preference by the herbivore. However, to my knowledge, this is the first time that the effect of pathogen-induced biochemical changes in plants on parasitoid behavior has been evaluated.

Recent studies suggest that activation of plant defenses by pathogens interferes with plant defenses against herbivorous arthropods and vice-versa. In light of these possible conflicts between the pathogen- and herbivore-induced plant defenses, the lack of studies on the interactions between insect and pathogen species, and the induced

chemical changes that such interactions have on the host plant has become apparent. In chapter 5, I investigated the possible production of volatile compounds by pepper plants at different times during infection by the pepper leaf spot bacteria, *Xanthomonas campestris* pv. *vesicatoria*, and evaluated the effect of simultaneous bacteria/herbivore challenge on the volatile emission by these plants. The level of the plant hormones JA and SA, which are believed to be involved in herbivore-and pathogen-defense signaling in plants, was measured in plants treated with XCV pepper race 3 (XCVP3) and BAW individually and in combination. In this study I found that pepper plants produced different volatile profiles in response to XCVP3, and BAW damage. Additionally, I found that plants under simultaneous attack by the bacteria and the insect were able to produce a volatile profile qualitatively similar, but quantitatively greater, than that produced by plants under insect damage alone during the 4 days following bacterial inoculation. However, after 4 days from bacterial inoculation, the amount of volatiles emitted by XCVP3-infected plants in response to BAW damage was significantly lower than that produced by healthy plants in response to BAW damage. Analyses of the levels of signaling hormones JA and SA in these plants revealed that the reduction in volatile production in response to insect damage was preceded by a significant increase in conjugated SA, and consequent release of methyl salicylate by plants under the combined bacterial/insect attack. Also, the cumulative JA increase in XCVP3/BAW plants was significantly higher than that of control plants, but did not differ significantly from those of plants damaged by either organism alone. The data presented herein support the idea of an orchestrated interaction between the levels of JA and SA to regulate the induction and emission of volatile organic compounds by pepper plants under bacterial- and insect

herbivore-attack. This is the first time in which the levels of both JA and SA have been correlated with plant emission of volatile compounds in response to individual and simultaneous pathogen- and insect herbivore- attack have been studied. Studies such as these are necessary to help unravel the complex interactions between defense pathways that help plants cope with multiple stress factors. However, further experiments are necessary to determine the JA/SA ratios necessary for volatile induction and/or inhibition in this plant-herbivore-pathogen system as well as others. Additionally, the hierarchy governing plant defense responses when under attack by pathogenic and herbivorous organisms merits further attention.

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BIOGRAPHICAL SKETCH

Yasmin Judith Cardoza was born on October 13, 1971 in Florida, Copan, Honduras, Central America. After graduating from high school, in December 1989, she applied and was granted financial assistance to attend the Panamerican School of Agriculture "El Zamorano" in Tegucigalpa, Honduras. She enrolled in a 3-year program at "El Zamorano" from January 1990 to December 1992, when she was granted an Agronomist degree.

In July 1993, Yasmin was accepted at the University of Florida. In December 1994, she obtained her Bachelor of Science degree from the Entomology and Nematology Department. During her undergraduate program, Yasmin worked part-time in the laboratory of Robert Heath and Nancy Epsky at the former Insect Attractants Laboratory of the USDA-ARS in Gaineville, FL, now CMAVE, where she obtained a full-time position upon graduation. During this year, Yasmin worked on a number of projects studying the biology, behavior and control of the pickle-worm moth, oleander moth, fall armyworm, pepper and sweetpotato weevils, and the Mediterranean and Caribbean fruit flies.

In November 1995, she was offered a graduate research assistantship to evaluate host-plant resistance to whiteflies under the supervision of Heather McAuslane. She graduated with a Master of Science from the University of Florida's Entomology and Nematology in May 1998.

After graduation, Yasmin obtained a practical training position in the laboratory of Dr. James Tumlinson at the Insect Chemistry Unit of the USDA-ARS/CMAVE. During this year, she studied the effect of biotic and abiotic factors on the release of volatile synomones by corn and cotton seedlings in response to herbivorous insect attack. In July 1999, Yasmin accepted a research assistantship to work on her Ph.D. program under the supervision of Dr. James Tumlinson. The area of research involved the chemical interactions among host plants, phytopathogens, insect herbivores, and parasitoids of the herbivores. Her projects included evaluation and identification of volatile distress signals emitted by plants under attack by insect herbivores and plant pathogens, and by their simultaneous attack on the host plant. Further studies involved evaluation of the effect of the pathogen-induced plant defenses on the host searching behavior by herbivore moths and their parasitoid wasps. Yasmin has been married for 9 years to Alonso Suazo-Calix, who also has his Ph.D. in Entomology, specializing in honeybee genetics.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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Professor of Entomology and
Nematology

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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This thesis was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 2002

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